Effects on cellular energy allocation and total oxyradical capacity in contamination exposed *Arenicola marina*

Kenneth J. Macrae

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University of Oslo, Norway

Department of Biology, Faculty of Mathematics and natural sciences,

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Dette prosjektet ble utført både hos NIVA og ved Universitetet i Oslo med henholdsvis Ketil Hylland og Merete Grung som veiledere.

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Abstract

Sediment in Frierfjord, Southern Norway is polluted by heavy metals, PAH, PCB, dibenzo-*p*-dioxins and dibenzofurans. Levels of toxicity in sediment cannot be determined through chemical analyses alone due to biological, chemical and physical interactions with the sediment and biota. This study aimed to assess toxicity through exposure of *Arenicola marina* to the sediment. Sub-lethal effects in oxidative stress resistance (total oxyradical scavenging capacity- TOSC) and alterations to the energy budget (cellular energy allocation- CEA) were determined and related to sediment exposure. In addition to contaminated sediment, two reference sediments with different grain sizes were collected from outer Oslofjord. Effects were measured after 1, 2, 4 and 8 weeks of exposure in a microcosm experiment. Differences in bioaccumulation factors and energy budgets were detected in two Frierfjord sediments with similar levels of contamination. This demonstrated discrepancies in bioavailability of sediment-bound contaminants from the same area highlighting the need to consider spatial heterogeneity in sediment assessments. Two control sediments also affected *A. marina* differently, possibly due to dissimilar particle size, organic content and/or estrogenicity. Two Contaminated sediments were found not to affect the TOSC for three oxyradical agents (peroxyl radicals, hydroxyl radicals and peroxynitrite anions). Effects were discovered in the CEA of the exposure group with the highest levels of bioaccumulated toxicants. Exposure to one of the contaminated sediments was found to cause temporary increases in all measured energy storage parameters (protein, carbohydrate, lipids). Exposure to contamination also resulted in decreased levels of lipids and total available energy during the final 4 weeks, relative to one control group. It was concluded that effects in the energy budget due to contaminated sediment, were not caused by suppression of oxidative stress resistance in *Arenicola marina*.

1. Introduction

1.1. Introduction

There is a close relationship between industry and marine ecosystems. A dependence on shipping has led to many factories being positioned close to harbours and estuaries. A number of industrial processes also use estuaries as a site for the disposal of effluents. In addition to direct releases to these water bodies, a significant proportion of atmospheric emissions are also deposited within estuaries. These contaminant sources have caused estuaries to be amongst the most polluted sites in the world (Byrne and O'Halloran, 2001). Estuaries are also ecologically important areas. They are nurseries for juvenile fishes, routes for migratory fishes and feeding grounds for many bird species. Compromises have to be made in many estuaries favouring industry over the environment but it is important to minimise consequences to marine life.

Frierfjord is an estuary in Southern Norway which has been the site of industrial processes including the production of magnesium, electronics, silicone, cement and other building materials. Magnesium refining was one major source of contamination due to the use of chlorine at high temperatures. These conditions cause the formation of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (Knutzen and Oeheme, 1989). During first decades of production, emissions of these contaminants in Frierfjord were amongst the highest of any point source in the world. Other industrial sources have released polyaromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB) and heavy metals, making Frierfjord among Norway's most contaminated estuaries.

Despite a cessation of the production of magnesium and increased control of emissions, restrictions are in place for the consumption of seafood from this area. Sediments are now suspected to be the major source of contamination to marine life within this estuary. The the Norwegian Pollution Control Authority (SFT), the Norwegian Institute for Water Research (NIVA) and the Norwegian Geotechnical Institute (NGI) will therefore establish the sources of contaminants and determine their toxicity.

Sediments have a relatively poor nutritional quality (O'Connor et al., 1998). Nevertheless, intertidal sediments provide highly productive habitats. Organic production in these areas is mainly based on the recycling of nutrients from terrestrial runoff. One further source is the sedimentation of particles which are trapped by the ebb and flow of tides. These nutrients are consumed by bacteria and deposit feeders which, in turn, provide food for higher organisms. Processes which have been found to trap nutritional particles within estuaries are also known to trap contamination (Murphy et al., 1998). There is a tendency of fat soluble compounds such as PAH, dioxins and PCB to be associated with suspended particulate matter in the water column. Sedimentation of contaminated particles is known to be a major sink for contamination in estuaries (Ridgeway and Shimmield, 2002).

Contaminants can negatively affect benthic environments through the replacement of nutrient recycling species with more tolerant species (Burton et al., 2003). These toxic compounds can also be recovered from the sediment and incorporated in food webs through the same route as nutrient recycling. Furthermore, many persistent contaminants become more concentrated at higher trophic levels through biomagnification (Kostamo et al., 2002). Toxicity within the sediment can thus be a determining factor for health of the ecosystem.

One ecologically important sediment dwelling species is the polychaete *Arenicola marina*. This species is ubiquitous along the coastlines of North West Europe. Investigations of *A. marina* have been undertaken since the early 19th century and as a result there is an excellent background for its biology. This polychaete has been described as a species well suited to biological testing due to low mortalities in control groups and high tolerances to a range of environmental parameters (Zebe and Schiedek, 1996; Kaag et al., 1998). *A. marina* has been found to be a useful tool in toxicity testing of sediments (Bat and Raffaelli, 1998). Sediment dwelling deposit feeders, such as *A. marina*, are exposed to contaminants through interstitial water and ingested sediment particles (Landrum, 1989). As such, information on both of these aspects of sediment toxicology can be gained through their use.

During the last two decades there has been an increased interest in sediment testing for toxicological purposes. It is well documented that sediments are sinks for contaminants. However, research has demonstrated the difficulty in extrapolating data from sediment concentrations to toxicological effects (O'Connor et al., 1998). Biotic and chemical interactions with the toxic agents are one of the main causes of this challenge. Sediment testing has the ability to account for all factors and interactions simultaneously without an in depth knowledge of contaminants or specific pathways (Byrne and O'Halloran, 2001). Toxicity testing of industrially contaminated sediments is currently the best method to determine effects on sediment dwelling organisms.

Toxicity tests are methods for the determination of chemical or physical damage to living organisms due to toxicants. Such tests are carried out in an attempt to estimate environmental damage from given sources or to establish emission concession levels. When animals are employed for toxicity testing it is vital to reduce or remove pain and suffering of the test subjects. The first toxicity tests relied on values such as the concentration required to kill 50% of the test subjects (LC50). However, such tests cause unnecessary suffering and have limited ecological relevance. One alternative for lethality testing is the use of effect markers. Effect markers are sub-lethal tests that are related to health of an organism or a population. One successfully employed effect marker test is the scope for growth (SfG) (Warren and Davis, 1967). SfG uses energy budgets to predict the fecundity. For this purpose respiration is measured as are ingested and egested matter. Despite successful usage, SfG has some limitations. It is labour intensive and difficult to measure for many organisms. To solve this issue, a more simple method of energy budget calculation has been proposed: cellular energy allocation (CEA) (De Coen and Janssen 1997). CEA measures energy reserves and compares them to a biochemically determined respiration for the test organism. The relationship between available energy and the rate of energy consumption is integrated into the value for CEA which is presented as joules per gram of organism. This effect marker can be applied in toxicity testing to determine the metabolic cost of tolerating toxic stress. CEA has been found to have comparable results to life tables, relevance at the population level for *Daphnia magna* and greater

sensitivity than SfG in *Neomysis integer* (De Coen and Janssen, 1997; Verslycke et al., 2004)

Measurements of an organism's ability to tolerate toxic stress are also possible through the use of effect markers. Total oxyradical scavenging capacity (TOSC) is a method to determine the ability of an organism to detoxicate oxidative stressors (Winston et al., 1998). Various attempts to measure individual antioxidants and relate these to protection from oxidative stress have been made (Doyotte et al., 1997; Cossu et al., 2000; Lionetto et al., 2003). However, many oxidants can be detoxified by multiple mechanisms. Deficiencies in one antioxidant may be compensated for by the presence of others. The TOSC methodology measures the capability of an organism to detoxicate a known quantity of oxidative agents. Oxidants (endogenous and exogenous) that supersede antioxidant defenses within an organism can damage biomolecules. As such, TOSC is a relevant method for determination of the resistance to oxidative stress with implications for the organism's health.

1.2. Aims

This study aimed to determine whether sediment-bound contaminants would affect sub-lethal effect markers in an inbenthic polychaete and to quantify changes over time. Two samples from Frierfjord were chosen to represent heavily contaminated sediments. Two relatively unpolluted samples from outer Oslofjord were chosen as control sediments. The controls were chosen to account for the effect of different aspects of sediment quality (particle size and organic content). The selected sub-lethal effects markers would quantify oxidative stress resistance (TOSC) and effects on the energy budget (CEA). Measurements will be taken after exposures of 7, 14, 28 and 56 days to determine trends in each of these over time. The following hypotheses were tested:

Arenicola marina exposed to contaminated sediments did not have altered energy budgets.

Energy budgets in *Arenicola marina* were not affected during the experimental period of exposure to contaminated sediment.

Oxidative stress resistance in *Arenicola marina* was not affected by exposure to contaminated sediment.

Oxidative stress resistance in *Arenicola marina* was not affected during the experimental period of exposure to contaminated sediment.

2. Background

2.1. *Arenicola marina*

2.1.1 Reproduction

A. marina reproduce in the autumn and it has been suggested that testing on this species should not be undertaken between the end of August and November (Northern Hemisphere) due to variation in results gained during this time (Kaag et al., 1998). Furthermore an ideal window between December and May for the collection of biomonitoring samples has been suggested. This is due to variation in different populations as recorded by other authors. The time frame for the exposure in this experiment was from 30th May when the polychaetes were purchased in from England, to the 6th August which was the final endpoint for the exposure.

2.1.2. Habitat and areas

Wild populations of *A. marina* are rarely reported in sediments with average particle size of less than 80 μm or greater than 200 μm (Longbottom, 1970). In the finer sediments this absence is due to the difficulty of maintaining a burrow. For the larger diameters the polychaetes have difficulty ingesting these particles. Within this range there is a positive correlation between biomass and the level of organic matter in the sediment. Population densities of up to 70 per square meter have been recorded (Longbottom, 1970).

2.1.3. Water balance

It has been determined that *Arenicola marina* is a slightly hyperosmotic osmoconformer meaning that it has little ability to adjust the water balance in the body (Oglesby, 1973). Alterations in salinity will thus cause temporary changes in concentrations of biomolecules relative to wet weight. In addition to this, handling of the polychaetes causes an increase in the frequency and length of time which the excretory sphincters are open (Chapman and Newell, 1947) and most conceivably in the rate at which water is lost from the organism. These facts are in some ways a contraindication as to the suitability of this species for biochemical measurements which are related to wet weight. Whole organism measurements in conditions with slightly lower salinity or greater stress will give the impression of a higher reading simply due to a more concentrated sample.

Nevertheless, standardised treatment of all samples will reduce variations due to this error.

2.2. Exposure of *Arenicola marina* to contaminants

2.2.1. Diet

Arenicola marina is described as a sub-surface deposit feeder (Tyler-Walters, 2006). During the summer months it feeds on a diet of bacteria and microalgae which are both present in the sediment which they ingest (Andresen and Kristensen, 2002). Bacteria contribute 3% to 7% of their minimum carbon requirement whilst microalgae can account for 42% to 370%. During the winter months these sources together make up around 50%. Andresen and Kristensen suggest digestible detritus as the source of the remaining 50%. However Retraubun et al. (1996) cast doubt over this hypothesis suggesting that the residency time in the gut is too short and that there is a lack of evidence of digested detritus in the faeces.

2.2.2. Absorption

In addition to ingestion, sediment dwelling organisms are also exposed to contaminants directly from the interstitial water (Landrum, 1989). Relative exposures to compounds through diet and absorption are dependant on water solubility. Substances which are more hydrophilic, such as lower molecular weight PAH are absorbed in greater amounts from interstitial water than through diet. The greatest proportion of high molecular weight PAHs are taken up through ingestion.

There is evidence that *A. marina* can absorb volatile fatty acids (FAs) through the epidermis (Holst and Zebe, 1984). It has been demonstrated that FAs are produced by anoxic respiration amongst microorganisms. However, it is not known if the levels of volatile FAs in the burrow reach levels high enough to give a concentration gradient sufficient to enable transfer into the polychaete. If this were the case then it would be a route of both nutrition and intake of fat soluble hydrocarbons such as larger PAH, PCB and dioxins.

2.3. Biomarkers and effect markers

Biomarkers are tools that can be used to relate levels of biochemical, physical or behavioural variation which can be measured within an organism to provide information on responses to one or more chemicals (Depledge et al., 1995). To use biomarkers, a knowledge of the mechanisms and responses of a compound are required. This is in opposition with principles of sediment toxicity testing where whole sediments are used because of the lack of knowledge of the sum of the parts. In this incidence the term biomarker must therefore be replaced by effect marker. This term refers to a biological response to a compound where effects can not be predicted or linked to individual mechanisms.

3. Materials and Methods

3.1. Exposure

3.1.1. Source of *Arenicola marina*

Arenicola marina were purchased from Seabait polychaete farm, Lynemouth, England. These polychaetes were packaged in a protective environment of activated charcoal and oxygen in an isopore box containing ice and sent via courier to NIVA's research station at Solbergstrand, Norway. The packages with *A. marina* were then placed into a receiving tank with local sea water for 30 minutes to allow the polychaetes to adjust to the temperature of the test conditions. *A. marina* were then carefully put into local uncontaminated sediment for a further 12 days to recover from their transport and to acclimatize.

3.1.2. Aquaria and water supply

Water used for the experiment came from a position close to the shore at NIVA's research station at Solbergstrand in the outer Oslo Fjord. Underwater pipes carried water up from a depth of 60m into the station where it was monitored for water temperature and salinity levels. For this experiment the variation in water salinity was 32.9 ppt to 35.4 ppt and the temperature was from 7.5 ºC to 9.2 ºC. Daily measurements are given in section 9.

The site of the exposure was a dark room within the research station. Benches with a system for oxygenation of the water (figure 1a) were used to provide a steady and consistent supply of water to all of the tanks. The filtered water entered the wet room through a pipe which opened and allowed the water to fall slightly through the air, saturating the water with oxygen before entering the header tank. From the header tank the water drained out through one of many hoses that emptied into exposure aquaria providing a fresh supply of oxygenated sea water.

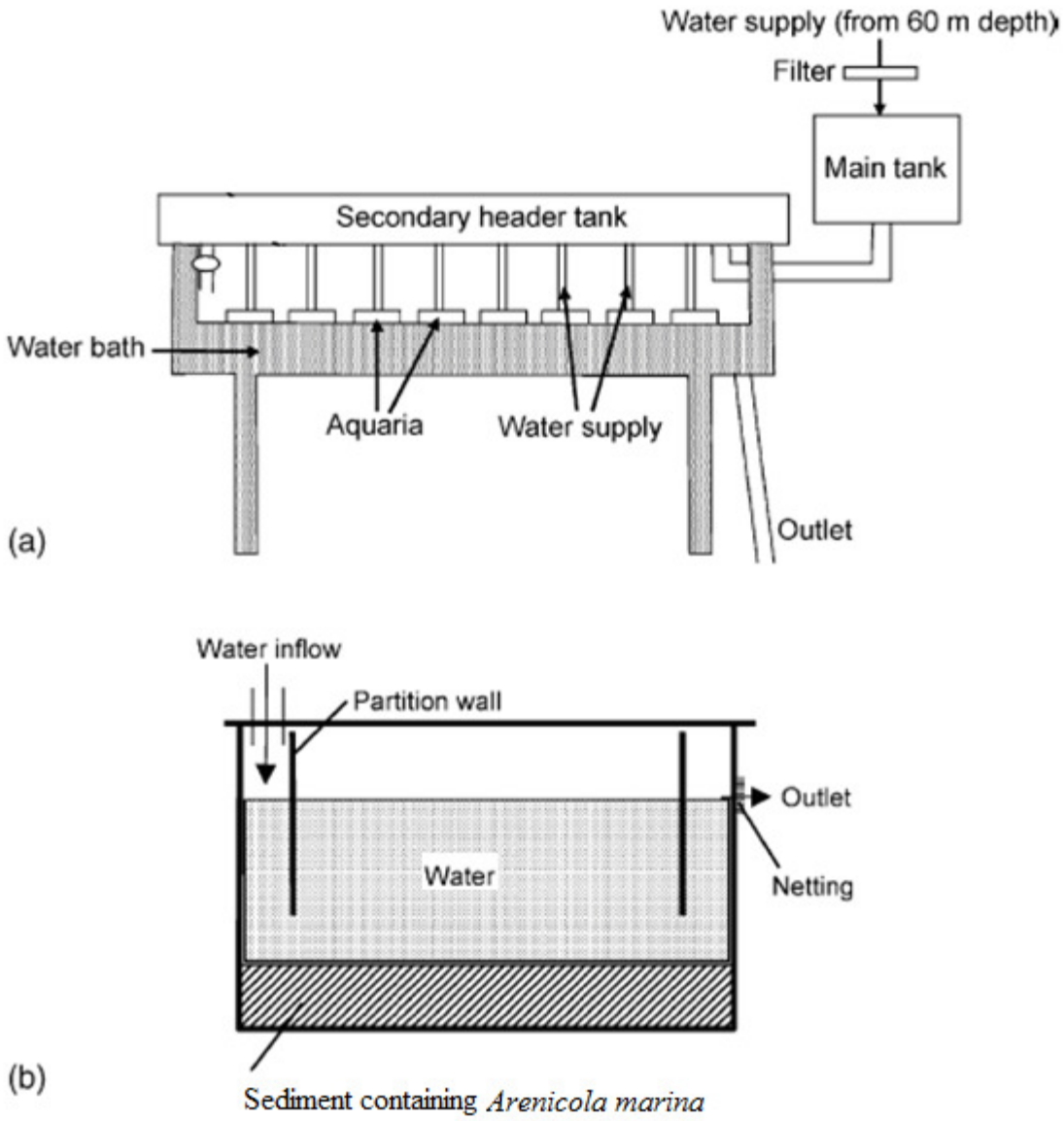

Figure 1. The setup of exposure aquaria and oxygenated water distribution system (a). Side on view of a test aquarium showing partitioning walls to convey oxygenated water over the sediment surface (b). (modified from Ruus et al., 2005)

Glass exposure aquaria (figure 1b) were used to house the polychaetes and the test sediments for the duration of the experiment. The aquaria were 15 cm x 15 cm x 25 cm and were partially divided into 3 parts. Water flowed into the aquarium from the top left in the figure from where it was forced down over the surface of the sediment by the 2 partition walls. Water left the tank through an overflow on the top right of the figure. This process ensured a good conveyance of oxygen rich water to the surface of the sediment.

3.1.3 Sediments

Sediments for four test sediments were collected from the positions marked on the map in figure 2 relative to Oslo harbour. Sediment A was taken from Elle, 'Sol.' (Solbergstrand) denotes sediment B whilst sediments C and D were taken from Frierfjord. A 0.1 m² box corer aboard the University in Oslo vessel Trygve Braarud was utilised for sediment sampling. Sediments were homogenised and 1 L was added to each of the 64 aquaria. Sediments were allowed to stand for 3 weeks before commencing the exposure. Data for the physical and chemical properties of the sediment is given in the section 4.2.

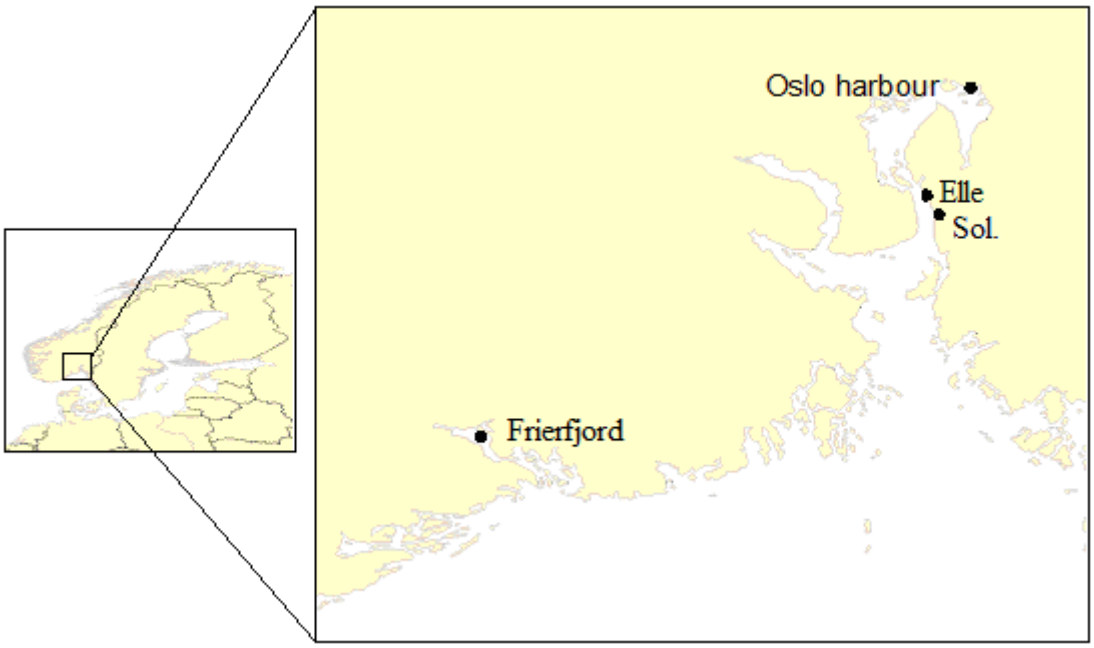

Figure 2. Map over the sites of sediment collection relative to Oslo harbour.

3.1.4. Harvesting

Prior to the commencing of the exposure, 8 polychaetes were processed to give baseline levels of all the parameters to be measured during analysis. Individuals were placed into 4.5 ml cryotube vials (Sigma Aldrich, Missouri, USA) and stored in liquid nitrogen for transport. Upon return to the laboratory the samples were places in a -80 ºC freezer until the time of analysis.

For each of the four sediments 16 polychaetes were added to separate aquaria. At 1, 2, 4 and 8 weeks four individuals were gently sieved from the sediment. The polychaetes were then processed as for the null samples.

3.1.5 Bioaccumulation tests

A study of the bioaccumulation of compounds was carried out simultaneously with the TOSC and CEA exposures. 4 polychaetes were taken at week zero, weighed and put into glass jars with aluminum foil under the lid to prevent contact with plastic. These were frozen at -40 ºC. Three further specimens were put into each of the four sediments for bioaccumulation testing. After 4 weeks of exposure, the bioaccumulation *A. marina* were removed from the sediment and stored using the same method as for the polychaetes at week null.

3.2. TOSC

A list of all reagents and solutions used for the laboratory analysis phase of this investigation is given in section 7.

3.2.1 Homogenisation

For each of the TOSC and CEA assays two quarters of each polychaete were utilised. With part 1 referring to the head and 4 being the rear end of the polychaete, parts 1 and 3 were used for the TOSC assay whilst parts 2 and 4 were used for CEA. This was to minimise any error due to differences in body parts.

The specific total oxyradical scavenging capacity was determined as in the method described by Regoli and Winston, (1999) with modifications for marine invertebrates as suggested by Camus et al., (2004). Batches of 16 polychaetes were randomly selected and thawed on ice. 1 part *A. marina* tissue was diluted with 4 parts potassium phosphate buffer (w:w) and the sample was homogenized using a Potter-Elvehjern glass / Teflon homogenizer. The homogenates were subsequently centrifuged at 50,000 G for 2h at 4 ºC to remove all cellular components from the cytosol.

Supernatants were divided in to 3 Eppendorf tubes with 100 µl for TOSC analysis and one with 50µl for the quantification of protein in the cytosol. Some of the remainder of supernatants were randomly selected and mixed together into a reference sample. This was put into 20 replicate Eppendorf tubes to be run with each batch of analyses. All tubes were kept on dry ice after homogenization and transferred to a -80ºC freezer as soon as possible.

3.2.2. GC setup

10 ml reaction glasses with stoppers containing rubber septa (Supelco, Bellefonte, PA, USA) were used to contain the reaction in which antioxidant levels of the tissue were established. A total of 1 ml of liquid was used in the reaction glasses comprising; 100 µl of tissue sample, 100 µl of α-keto-γ-methiolbutyric acid (KMBA) and either 100 µl or 200 µl of solution giving a specific type of free radical or free radical source. The rest of the 1 ml was made up by potassium phosphate buffer. For the peroxyl radical, glutathione was employed as a positive control. No positive control was used for hydroxyl radicals or peroxynitrite anions. An overview of the volumes of each reagent is given in section 7.3.

Free radicals which are present in the solution oxidize KMBA to ethylene gas. The presence of antioxidants was measured through the inhibition of this process. Ethylene gas was quantifiable by measurement with a 5890 Hewlet Packard Series II GC with flame ionization detector and a Supelco SPB-1 fused silica capillary column (30 m x 0.32 mm x 0,25 µm). Hydrogen, at a flow rate of 1 ml per minute was the carrier gas and a split ratio of 1 : 20 was used to measure the volumes of ethylene gas which were produced when KMBA reacted with free radicals in the glass vial. The program GC chem. station, (Rev A.09.01 (1206), copyright Agilent technologies, 1990-2001) was employed to analyze the readings from the GC and to calculate the areas under the peaks.

3.2.3. Calibration and reference sample

It was desirable that the value of the sample was approximately 50% of the negative control and that most of the samples were within 20 – 80%. For the peroxyl radical samples, a measurement of ethylene gas higher than the positive control (GSH) was also preferable. These criteria are met through dilutions which were calculated in a calibration run carried out on a reference sample made from a mixture of *A. marina* tissue. This reference sample was also run together with each batch of samples to ensure continuity and aid quality control of the data.

3.2.4. Gas chromatography (GC)

The reaction vials were initially put on ice and allowed to cool down for at least 5 minutes before the reagents were added. First the buffer was added to the reaction vial then the sample. Next, at exactly 30 second intervals, the remaining reagents were added to each vial. Immediately, the top was put on and tightened and the glass was shaken and put into a water bath set to 35 ºC. After exactly 24 minutes 200 µl of gas was taken from the head space using a gastight syringe (Hamilton, Bonaduz, GR, Switzerland) and the gas was injected into the GC. Three further measurements of gas from each vial were made at exactly 12 minute intervals giving four measurements in total.

3.2.5. Protein measurement

Eppendorf tubes containing 50 µl of sample which was prepared with the TOSC half of the polychaete were first diluted using Tris buffer at a ratio of 1 : 5 (v:v). Samples were already diluted four times giving a total twenty times dilution.

The prepared samples were then plated out on 96 well plates with 4 replicate wells for each. Each well contained 10 µl of sample, 25 µl of reagent A and 200 µl of reagent B. The plate was shaken by hand after addition of the chemicals. Reagents A and B were both at room temperature at the time of addition. The plate was read using a spectrophotometer (Molecular Devices Thermomax plate Reader, Sunnyvale, USA) at 750 nm with the data being processed by the Softmax Pro Software (Molecular Devices Corp, Sunnyvale, USA).

Accurate protein levels were determined through comparison to standard samples of a known concentration. For this purpose a dilution sequence was established with the following concentrations of γ-globulin in solution: 1470 mg/ml, 735 mg/ml, 368 mg/ml, 184 mg/ml, 92 mg/ml, 0 mg/ml.

4 replicates of each of these concentrations were measured together with each batch. The contents of the wells were the same as for the samples but with 10 µl of standard instead of a sample.

3.2.6. Calculation

The amount of ethylene gas produced in each reaction vial was plotted against time, and the area under the curve was calculated for samples (SA) and the controls (CA). Equation (1) was applied to calculate the experimental TOSC. This value was then multiplied by the dilution factor and divided by the amount of protein in the cytosol. Measurements are expressed as TOSC units per mg protein. Higher values demonstrate a greater ability of the sample to scavenge oxyradicals.

(1) $$TOSC = 100 - (\int SA / \int CA \times 100)$$

(Regoli and Winston, 1998)

3.3. CEA

3.3.1. Homogenisation

The remaining two quarters (sections 2 and 4) of the polychaete were thawed on ice, weighed and diluted 20x with the homogenisation buffer (w:w). Samples were homogenised using the same equipment and procedure as described for the TOSC analysis. The homogenates were centrifuged at 3000 G for 10 minutes and the supernatant was divided into Eppendorf tubes as follows;

300 µl for protein and carbohydrate measurement
200 µl for lipid measurement
150 µl for electron transfer system (ETS) analysis

2 replicates of the 300 µl and 200 µl Eppendorf tubes were made to minimise data loss due to any possible problems with the technique. All of the tubes were kept at -80 ºC prior to use. The remainder of the first batch of samples were mixed together and split into 150 µl and 300 µl samples. The 150 µl sample was used as a reference for ETS and the 300 µl sample was used as a reference for both protein and carbohydrates. These were also frozen before use.

3.3.2. Electron transport activity (ETS)

The electron transport activity was employed as an estimation of respiration in the specimens and was measured based on the technique proposed by King and Packard (1975). The samples to be analysed for ETS were not refrozen but were measured as soon as possible after centrifugation. 96 well microtest plates were utilised to measure multiple absorbencies simultaneously. Each well was filled with 100 µl of BSS, 50 µl NAD(P)H-solution and 50 µl of sample. Immediately prior to measurement, 100 µl of INT was added to each well and the plate was gently shaken by hand. 4 replicates were used for each sample and for the reference sample. A plate spectrophotometer which was set to 490 nm and in a temperature of 20 ºC took measurements every 15 seconds over 10 minute period. Data was analysed using the Softmax Pro Software.

3.3.3. Carbohydrates and protein preparation

Measurements for proteins and carbohydrates were taken from the same frozen 300 µl sample. The samples were thawed on ice before the addition of 100 µl of 15% TCA and vortex mixing followed centrifuging at 13000 rpm for 5min. The supernatant was transferred to another Eppendorf tube and the process was repeated one more time with 100 µl 5% TCA added to the pellet. Again the supernatant was transferred to the Eppendorf tube containing the first supernatant fraction. Analyses for carbohydrates was carried out on the supernatant whilst the pellet was used to establish protein concentration.

3.3.4. Total protein

Protein was measured according to the Lowry method (Lowry et al., 1951). Pellets were resuspended in 500 µl of 1 N NaOH. The samples were then heated at 60 ºC for 30 min. After heating the solution was neutralised by the addition of 1.67 N HCl. These were analysed using the same procedure as for the cytosol proteins in (section 3.2.5.).

3.3.5. Carbohydrate

The following reagents were added to a 96 well microtest plate together in the given order for the measurement of carbohydrates. 4 replicate wells were used for each sample.

- 50 µl of sample and TCA supernatant
- 200 µl sulphuric acid
- 50 µl 5% phenol in distilled water

The plate was then gently shaken and left to stand in a fume cupboard for 30 minutes. Absorbency at 490 nm was then measured using the spectrophotometer and Softmax program.

For the purpose of calibration, a standard of glycogen from bovine liver was used in place of the 50 µl of sample in 4 replicate wells. A dilution sequence was used to attain the following concentrations: 1.5 mg/ml, 0.75 mg/ml, 0.375 mg/ml, 0.1875 mg/ml, 0.09375 mg/ml and 0 mg/ml

It was determined during the reading of the plates that the upper and lower rows in the 96 well microtest plate gave lower readings for this assay than the other 3 replicates for each sample and they were removed. It is believed that these wells could have had significantly lower exposure to the heat generated by the exothermic reaction than those in the remainder of the plate. One further possibility could be that they were at a lower temperature by the end of the 30 minute standing time than those in the middle of the plate. This phenomenon did not, however, effect the blank and lowest concentration standard which were also on an outer edge. These were therefore used in the calculation of the standard curve.

3.3.6. Lipid

Fat levels in *Arenicola marina* were measured based on the technique employed by Bligh and Dyer (Bligh and Dyer, 1959). For the measurement of lipid content, Eppendorf tubes containing 200 µl were thawed on ice before the addition of 500 µl of chloroform and vortex mixing. 500 µl methanol and 250 µl water were added followed by further vortex mixing. The samples, together with 2 reference samples, were centrifuged at 14,000 rpm for 5 minutes. After centrifuging, 2 liquid phases were clearly visible with a thin solid layer between them. The lipids to be measured in this analysis were present within the lower, chloroform layer.

To remove a volume of this liquid it was necessary to tilt the Eppendorf tubes about 30 degrees and push the pipette tip under the solid layer. 100 µl of the chloroform phase was collected and put into glass tubes already containing 500 µl sulphuric acid. The sulphuric acid and chloroform were mixed carefully using a 1 ml pipette and incubated at 200 ºC for 15 minutes.

On removal from the oven, the glasses were left to cool to room temperature before the careful addition of 1ml distilled water and mixing with a 1 ml pipette. The absorbency at 340 nm was measured for 4 replicates of 200 µl of each sample and the reference sample. The spectrophotomerter used was a Perkin Elmer Victor 1420 multilabel counter (Massachusetts, USA).

Lipid concentrations were calculated through comparison with solutions containing the following volumes of 3 mg/ml tripalmitine solution made up to 600 µl with sulphuric acid: 100 µl, 50 µl, 25 µl, 12 µl, 6.25 µl and 0 µl.

3.3.7. Calculations for CEA

The energy consumed (Ec) was calculated using the number of moles of formazan produced which is given by equation (2) and dividing this figure by 2 to give the moles of oxygen consumed.

(2) Absorbance units= 15900 / M x L

(De Coen and Janssen, 1997)

In this equation M is the molarity of formazan and L is the distance in cm of sample the light passed through before reaching the sensor in the spectrophotometer.

The energy available (Ea) was calculated using the following enthalpies of combustion: protein 24 KJ/g, carbohydrates 17.5 KJ/g and fat 39.5 KJ/g (Gnaiger, 1983). The sum of these combined energies provided the total energy available for each polychaete.

Calculations for CEA were made by comparison of the available energy and consumed energy. Changes in available energy were calculated between each endpoint and expressed as difference per week. These were compared to the amounts of energy consumed between endpoints, also expressed per week, using equation (3)

(3) $$\text{CEA (J / g)} = \frac{\int_{t-1}^{t} \text{Ea x dt} - \int_{t-1}^{t} \text{Ec x dt}}{\text{dt}}$$

(De Coen and Janssen, 1997)

3.4. Chemical and particle size analyses

The following analyses were carried out by the chemical analysis section at NIVA's head office in Oslo.

3.4.1. Analysis for polyaromatic hydrocarbons (PAH) in *Arenicola marina*

The biological material was homogenised, deuterated PAH internal standards were added and samples were hydrolysed to remove excess fat. Samples were extracted with n-pentane and dried over sodium sulphate. The extract volume was reduced and purified by gel permeation chromatography (GPC). Where necessary, samples were further cleaned up using silica columns eluted with dichloromethane. The extracts were analyzed by gas chromatography mass spectrometry using selective ion monitoring (Hewlet Packard

6890- Hewlet Packard 5973 MSD). The gas chromatograph (GC) was equipped with a 30 column (J&W Scientific DB-5MS) and coated with 5% polysiloxane (0.25 mm inner diameter and 0.25 µm film thickness). The initial column temperature was 60 ºC which after 2 minutes was increased to 250 ºC at a rate of 7 ºC per minute. The rate of temperature increase was further raised to 15 ºC per minute after 250 ºC until a final temperature of 310 ºC. The injector temperature was 300 ºC and operated in splitless mode, the transfer line was at 280 ºC and the ion source temperature was 230 ºC. The flow rate was 1.2 ml per minute. Quantification of individual components was achieved through use of the internal standards.

3.4.2. Analysis for PAH in sediments

The sediment was homogenised and internal standards were added. Samples were extracted by accelerated solvent extraction (ASE) using DCM/cyclohexane (1:1) at a temperature of 100 ºC and a pressure of 2000 psi. Further clean up and analysis of the extracts were as described for the analysis of PAH in *A. marina*.

3.4.3. Analysis for polychlorinated biphenlyls (PCB) in *Arenicola marina*

Biological material was homogenised and labelled internal standards were added. Samples were double extracted by ultrasonic extraction using cyclohexane:acetone (4:3) for 3 minutes The extracts were washed with 0.5% saline solution. The extract volume was then reduced under nitrogen and the extracts were purified by gel permeation chromatography. Extracts were acid treated with concentrated sulphuric acid and subsequently analysed by gas chromatography with electron capture detection (Agilent technologies 6890). The GC was equipped with a 60 m column (J&W Scientific DB-5) coated with 5% polysiloxane (0.25 mm inner diameter and 0.25 µm film thickness) and the inlet was splitless. The initial column temperature was 90 ºC and after 2 minutes this was increased to 180 ºC at a rate of 10 ºC per minute. Subsequently the column temperature was further increased to 270 ºC at a rate of 2 ºC per minute and finally 310 at a rate of 20 ºC per minute. The injector temperature was set to 255 ºC and the detector temperature was 285 ºC. The flow rate was 1 ml per minute. Quantification was achieved through the use of internal standards.

3.4.4. Analysis for PCB in sediment

The sediment was homogenised and internal standards were added. Samples were extracted by accelerated solvent extraction (ASE) using DCM/cyclohexane (1:1) at a temperature of 100 ºC and a pressure of 2000 psi. Further clean up and analysis of the extracts were as described for the analysis of PCB in *A. marina*.

3.4.5. Metal analysis in sediment and *Arenicola marina*

Metal concentrations were determined as in Ruus et al. (2005). The polychaete samples were digested in 5-10 ml HNO_3 in a microwave oven then diluted with 50-100 ml distilled, deionised water. All metals apart from mercury were measured with the use of inductive plasma/mass spectrometry in a a Perkin-Elmer Sciex ELAN 6000 ICP-MS (Perkin Elmer, Wellesley, MA, USA). This was equipped with a Perkin-Elmer auto-sampler AS-90 and a Gibson 312 peristaltic pump (Gibson Inc., Middleton, WI, USA). Sediments were analyzed on a Perkin-Elmer Optima 4300 PV. Analysis of mercury was undertaken by cold vapor atomic absorption with the use of Perkin-Elmer Fims 400.

3.4.6. Particle size analysis

A sieve with a diameter of 63 μm was used to establish the proportion of particles over this size.

3.4.7. Total organic carbon TOC

A Carlo Erba 1106 elemental analyser (Thermo Electron Corp., Milan, Italy) was used to determine the organic content of sediments after combustion at 1800 ºC and acidification was utilised to remove the inorganic fraction. By the subtraction of the inorganic carbon from the total carbon the total organic carbon was determined.

3.5 Bioassays

The following analyses were carried out by the ecotoxicology section at NIVA's head office in Oslo.

3.5.1. Yeast estrogen screen (YES) and Yeast androgen screen (YAS)

The yeast estrogen screen was carried out based on the method of Routledge and Sumpter (1996). The yeast androgen screen was carried out as described by Sohoni and Sumpter (1998) *Saccharomyces cerevisiae* with genes for the human estrogen receptor/ human androgen receptor inserted into the genome (Galaxo- Wellcome, plc, Stevenage, Herts, UK) were utilised for this test. Plasmids containing estrogen or androgen responsiveness sequences controlling the lac-Z gene (encoding β-galactosidase) were also present in the yeast.

Samples to be tested were diluted in DMSO and added in duplicate together with the assay medium (200 µl) consisting of growth medium, chlorophenol red β-galactopyranoside and yeast cells. Estrogenic / androgenic substances were detected by a change of colour in the yeast cultivation medium, from yellow to red. The colour change is caused by the expression of the lac-Z gene which encodes the enzyme β-galactosidase. Estradiol / dihydrotestosterone equivelents were calculated by comparison with colours produced by known concentrations of estradiol / dihydrotestosterone. The YES was incubated at 32 ºC for 72 hours with a two minute shake after each 24 hour period. The YAS assay was incubated at 32 ºC for 24 hours, 28 ºC thereafter and read at 48 hours.

Anti-estrogenic and anti-androgenic activity were achieved through the addition of known concentrations of estradiol / dihydrotestosterone to the yeast together with the sample to be tested. Reductions in the expression due to the hormones was used to calculate inhibition.

Quantification of expression was achieved colourimetrically using a UV-vis plate reader (Perkin Elmer Victor 1420 multilabel counter) at an absorbance of 540 nm for colour and 620 nm for turbidity.

3.5.2. DR CALUX bioassay for dioxins and dioxin like compounds

The DR CALUX bioassay was carried out on exposure sediments using the procedures outlined in (Garrison et al., 1996). The CALUX cells consist of the mouse hepatoma (H4IIE) cell line that has been transfected with the luciferase reporter gene (pGudLuc)

from the firefly, *Photinus pyralis*. The cells were grown in MEM Alpha growth medium (Invitrogen, Auckland, NZ) with 10% Newborn Calf Serum (Invitrogen, Auckland, NZ) in 96-well plates before exposure. The sediment samples, dissolved in Dimethyl Sulphoxide (DMSO), and a 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Supelco, Bellefonte, PA, USA) standard curve were added to the cells which were exposed for 24 hours. After cell wash and lysis of cells, the luciferase enzyme was added, Steadylite (Perkin Elmer, Waltham USA). The emitted light from the luciferase activity was determined using an automated luminometer (1450 LSC Luminescence Counter, Perkin Elmer) and interpolated with the TCDD standard curve to produce the TEQ (TCDD equivalent) CALUX values for the sample extracts.

3.6. Statistical methods

For TOSC and CEA parameters, comparisons were made between the four exposure sediments at each of the endpoints. Statistical comparisons were also made between each of the subsequent endpoints, including week null, for each of the sediments.

In addition, differences were calculated between the values for each week for the energy storage parameters and ETS. For these the median values of one week were subtracted from the values from the subsequent week. Statistical comparisons were made between sediments at each of the endpoints for these differences.

Statistical calculations were made for TOSC oxyradical sources and CEA parameters using the statistics program R. Samples were analysed for non homogeneity of variance using Levene's test. Data which were found to be homogeneously distributed were analysed by two-way ANOVAs where possible. One-way ANOVAs were used where homogeneity was only found for one factor. A Tukey's test was used to establish which groups were significantly different. For non homogenous data a Kruskal Wallis test was used with a Mann Whitney pairwise test to determine which samples were significantly different. For all comparisons p was set to 0.05 and a Bonferroni correction was made to the Mann Whiteny test to account for 3 repeated comparisons.

4. Results

4.1. Sediment quality

Elle sediment was found to have the lowest level of total organic carbon and the highest particle size proportion of greater than 63 µm (Table 1). The highest TOC was discovered in Frierfjord sediment and the smallest proportion of particles above 63 µm was found in the Solbergstrand sediment.

Table 1. Levels of total organic carbon (TOC) in mg per gram d.w. and the proportion of particles with a diameter over 63 µm (p.p.). TOC is the value from a single sediment sample and p.p. is presented as the mean and standard deviation of two samples.

Sediment	TOC	p.p. > 63µm (%)	Std. dev.
A	12.6	56.5	0.46
B	21.7	3.1	0.73
C	40.8	9.8	0.42
D	45.3	9.9	1.28

4.2. Sediment contaminants

4.2.1 Concentrations of chemicals

Levels of various contaminants with known environmental toxicities were measured in each of the test sediments (tables 2 to 4). For each of the parameters measured sediments C and D had greatly elevated levels in comparison with control sediments A and B. This indicated that the Frierfjord sediment was indeed more contaminated than the control sites at Elle and Solbergstrand.

Table 2. Levels of metal contamination in each sediment. Concentrations are the mean and standard deviation of three replicate samples (expressed as µg / g d.w.)

	Cadmium		Copper		Mercury		Lead	
Sediment	Average	Std. dev.	Average	Std. dev.	Average	Std. dev.	Average	Std. dev.
A	<0.2		23.93	1.4	0.20	0.02	38.00	0
B	0.13	0.03	35.60	0.2	0.24	0.01	65.33	1.5
C	2.33	0.06	54.90	1.0	3.67	0.07	159.67	2.1
D	2.43	0.12	55.90	5.4	3.32	0.51	148.67	9.1

Table 3. Levels of PAH contamination in each sediments. PAHs are grouped by structure. 1-2 ringed PAH, pyrene, 4-6 ringed PAH and the sum of all PAH are given. Concentrations are the mean and standard deviation of three replicate samples (expressed as ng / g d.w.).

	1 - 2		Pyrene		4 - 6 (not pyrene)		Sum PAH	
Sediment	Average	Std. dev.	Average	Std. dev.	Average	Std. dev.	Average	Std. dev.
A	408	119	207	47	488	63	1384	270
B	364	25	183	15	1024	53	2063	121
C	4595	87	2200	0	6373	671	18392	1007
D	4052	27	2100	0	6663	67	18162	157

Table 4. Levels of PCB congener 209 contamination in sediments from each group. Concentrations are the mean and standard deviation of three replicate samples (expressed as ng / g d.w.).

	CB209	
Sediment	Average	Std. dev.
A	<0.5	
B	<0.5	
C	683	6
D	680	44

4.2.2. Sex hormonal agonist and antagonist assays

For the hormonal agonist assays only sediment A contained a concentration above the detection level for YES. No (table 5). For antagonist effect assays, sites C and D were found to have the greatest levels.

Table 5. Levels of hormonal disruptors in each sediment. Results are averages and standard deviations of 2 replicates for sediments A and B and 4 replicates for C/D. Concentrations are expressed as µg / g d.w.

	YES estradiol (E2)		YAS Dihydrotestosterone	Anti-YES 4-Hydroxytamoxifen		Anti-YAS Flutamide	
Sediment	Equivalent	St. dev.	(DHT) equivalent	equivalent	St. dev	Equivalent	St. dev
A	88	1	<0.4	<1.20		<4.80	
B	<0.004		<0.4	<1.20		<4.80	
C/D	<0.004		<0.4	1050	210	557	42

4.2.3. DR CALUX

The DR CALUX assay is a screen for dioxins and dioxin like PCBs. It was found that both heavily contaminated sediments (C and D) contained 1936 ±139 pg /g of 2, 3, 7, 8-tetrachlorodibenzodioxin equivalents (TEQ, mean and standard deviation of four samples). Neither of the control sediments had a TEQ above 0.6 pg/g d.w.

4.3 Bioaccumulated contaminants in *Arenicola marina* tissue

Levels of various contaminants which bioaccumulated in *Arenicola marina* were determined. Polychaetes exposed to each of the sediments and null sample individuals were analyzed. Results of these analyses are displayed in tables 6 to 9. Levels for each of the metals and pyrene were highest in the null group. Group C contained higher levels of bioaccumulated cadmium, lead and each of the PAH groups than group D. In each incidence, groups A and B had lower levels of PAH than sediments C and D. PCBs were only detected in Frierfjord exposed *A. marina.*

Table 6. Levels of bioaccumulated metal in *Arenicola marina*. Concentrations are the mean and standard deviation of three replicate samples (expressed as µg / g w.w.).

	Cadmium		**Copper**		**Mercury**		**Lead**	
Group	**Average**	**Std. dev.**	**Average**	**Std. dev.**	**Average**	**Std. dev.**	**Average**	**Std. dev.**
Null	0.08	0.009	2.21	0.79	0.08	0.010	1.54	0.82
A	0.05	0.007	0.64	0.07	<0.03		0.46	0.18
B	0.04	0.006	0.75	0.08	<0.03		0.41	0.13
C	0.05	0.019	0.90	0.45	0.04	0.019	0.89	0.54
D	<0.03		0.57	0.05	0.03	0.003	0.41	0.10

Table 7. Levels of bioaccumulated PAH in *Arenicola marina*. PAHs are grouped by structure. 1-2 ringed PAH, pyrene, 4-6 ringed PAH and the sum of all PAH are given. Concentrations are the mean and standard deviation of three replicate samples (expressed as ng / g w.w.).

	1 - 2		PYR		4 - 6 (not PYR)		**Sum PAH**	
Group	**Average**	**Std. dev.**	**Average**	**Std. dev.**	**Average**	**Std. dev.**	**Average**	**Std. dev.**
Null	6.85	2.94	9.03	2.78	13.98	2.84	25.93	4.54
A	2.75	0.44	4.53	0.60	8.73	1.51	15.97	3.82
B	2.45	0.82	2.60	1.01	9.63	3.98	16.63	8.82
C	11.77	4.08	12.03	4.32	26.03	6.53	78.20	23.83
D	6.70	1.71	5.80	1.01	12.90	2.69	35.23	8.45

Table 8. Levels of bioaccumulated PCB in *Arenicola marina*. Selected congeners are given. Concentrations are the mean and standard deviation of three replicate samples (expressed as ng / g w.w.).

	PCB101		**PCB138**		**PCB153**		**Sum PCB**	
Group	**Average**	**Std. dev.**	**Average**	**Std. dev.**	**Average**	**Std. dev.**	**Average**	**Std. dev.**
Null	<0.5		<0.5		<0.5		<0.5	
A	<0.5		<0.5		<0.5		<0.5	
B	<0.5		<0.5		<0.5		<0.5	
C	1.30	0.35	0.84	0.34	1.23	0.41	3.56	1.39
D	1.02	0.24	0.41	0.27	1.12	0.24	2.38	0.90

4.4 Weight of whole polychaete

For simplicity, the term 'endpoint' will henceforth be used to describe time of removal of each polychaete from the sediment. Due to the nature of the investigation it was not possible to sample the same polychaetes at multiple endpoints. However, all individuals exposed to each of the four sediments were subject to identical conditions throughout the investigation. It will therefore be assumed that measurements made of *A. marina* harvested at a one endpoint represented what measurements of the rest of the same group would have been had they been harvested at that time (e.g. lipid levels for group A, harvested at week 1, were the similar to levels of for all group A polychaetes after a one week exposure).

The weights of individual polychaetes are given in figure 3. It was established that sediment type and exposure time had significant effects on weight in a two-way ANOVA (sediment; p=0.001, weight p= 0.031). A reduced weight in group A relative to sediments B (p = 0.015) and D (p = 0.001) was detected by a Tukey's test. In addition week one was found to be different to weeks 2 (p=0.016) and 4 (p=0.013). This was due to lower values for groups B, C and D at weeks 2 and 4 than week 1.

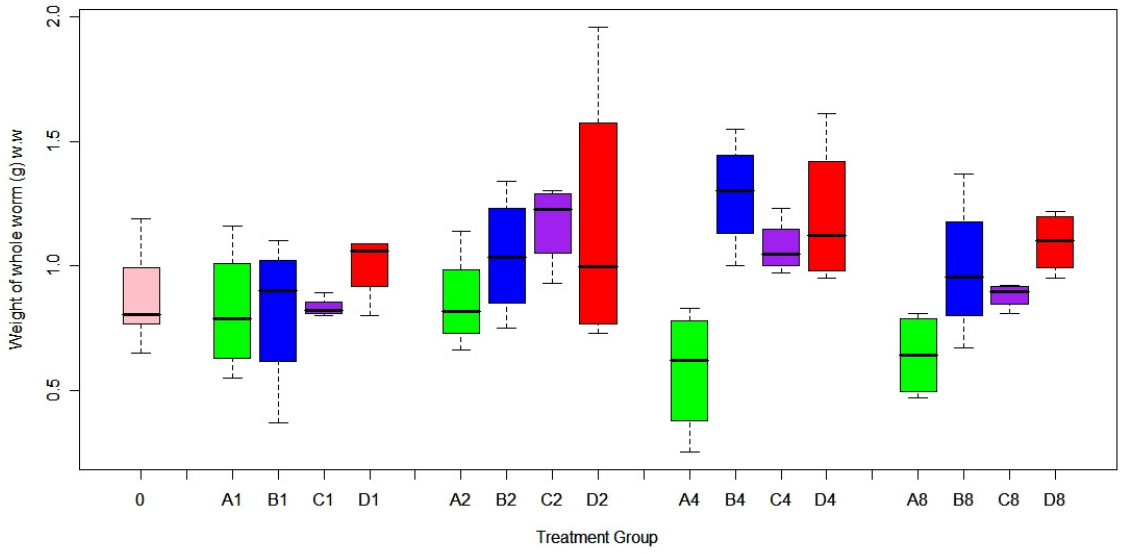

Figure 3. Weight of *Arenicola marina* after exposure to test sediments. A, B, C and D indicate exposure sediment. Numbers 0, 1, 2, 4 and 8 indicate the length of exposure in weeks. Median, quartiles, maximum and minimum values are presented.

4.5. Total oxyradical scavenging capacity (TOSC)

4.5.1. Peroxyl

Total oxyradical scavenging capacities of *A. marina* tissue against peroxyl radicals are displayed graphically in figure 4. No significant differences due to sediment or exposure time were detected using a two-way ANOVA.

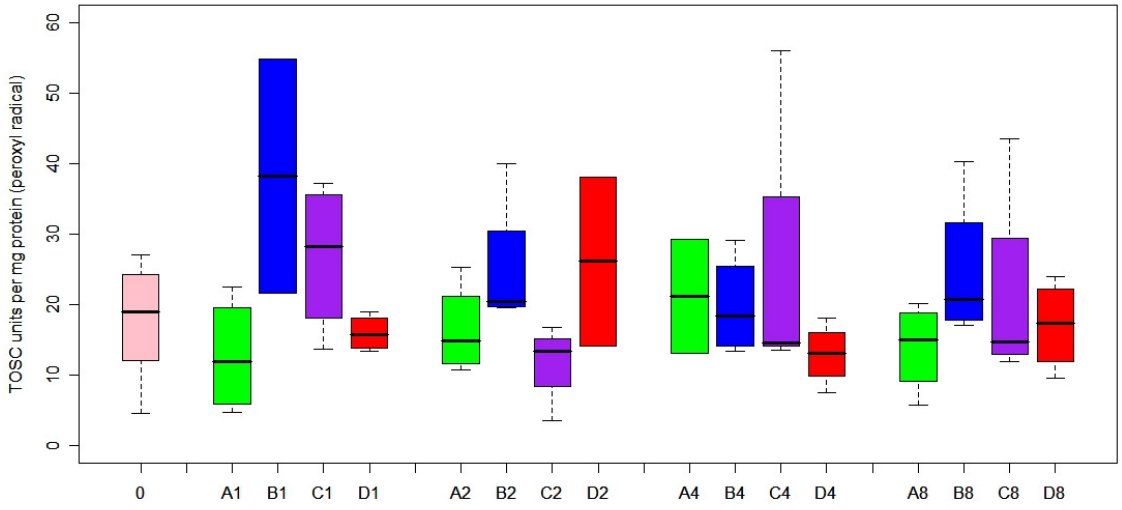

Figure 4. Total oxyradical scavenging capacity for peroxyl radicals in *Arenicola marina* after exposure to test sediments. A, B, C and D indicate exposure sediment. Numbers 0, 1, 2, 4 and 8 indicate the length of exposure in weeks. Median, quartiles, maximum and minimum values are presented.

4.5.2. Hydroxyl

The capacity of exposed *A. marina* to scavenge hydroxyl radicals is presented graphically in figure 5. No effects due to exposure time were found on TOSC using a one-way ANOVA. Sediment type was also found not to have any effect in a Kruskal Wallis analyses. The greatest variance in results was found in the null exposure group.

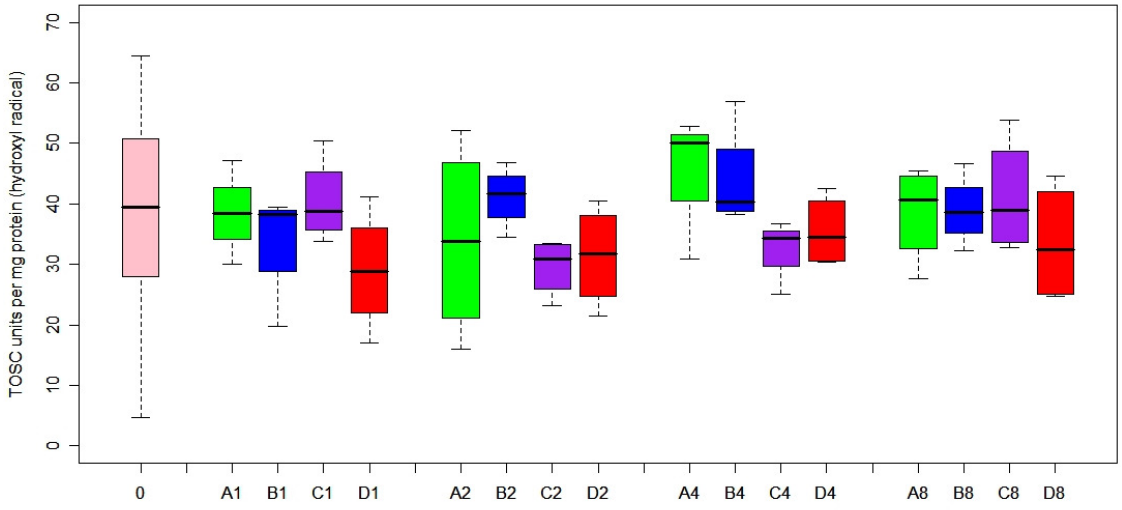

Figure 5. Total oxyradical scavenging capacity for hydroxyl radicals in *Arenicola marina* after exposure to test sediments. A, B, C and D indicate exposure sediment. Numbers 0, 1, 2, 4 and 8 indicate the length of exposure in weeks. Median, quartiles, maximum and minimum values are presented.

4.5.3. Peroxynitrite

Again the null group was determined to have the greatest variance with differences over an order of magnitude for peroxynitrite anion generated oxyradical stress (figure 6). No statistically significant effects were found due to sediment exposure or duration in Kruskal Wallis tests.

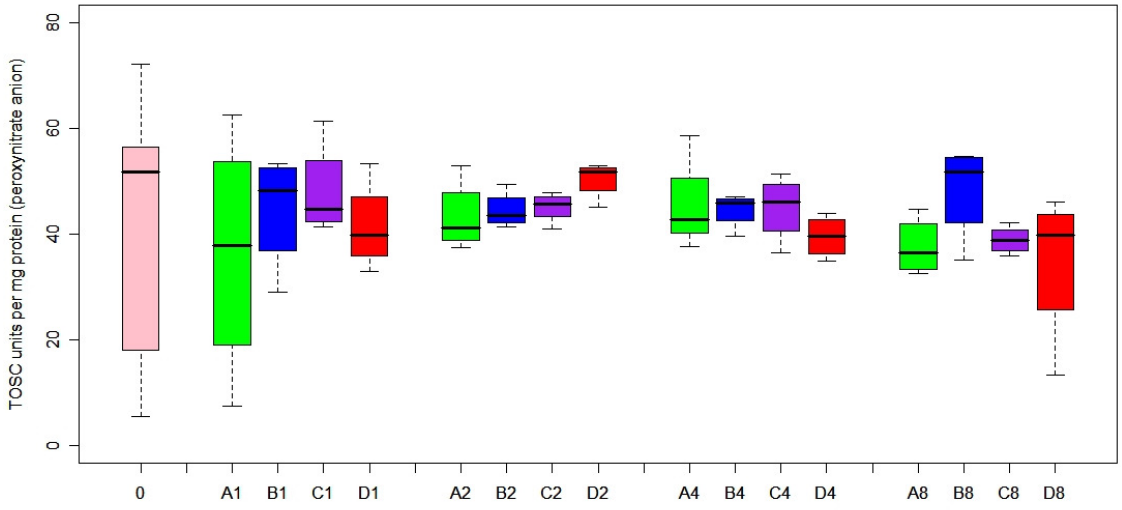

Figure 6. Total oxyradical scavenging capacity under exposure to peroxynitrite anions in *Arenicola marina* after exposure to test sediments. A, B, C and D indicate exposure sediment. Numbers 0, 1, 2, 4 and 8 indicate the length of exposure in weeks. Median, quartiles, maximum and minimum values are presented.

4.6. Cellular energy allocation

Each of the constituent parameters, in addition to the cellular energy allocation, were examined for significant differences between sediments and between endpoints. As already mentioned it was assumed that measurements taken at each endpoint were representative of all polychaetes exposed to that sediment at that moment in time. Using this assumption, differences were calculated between endpoints for each group. This gave an expression of the effects of that period alone. For this, the median value for the previous endpoint was subtracted from values for each treatment at a given endpoint (e.g. the median of week 2 was subtracted from values from week 4). The differences between sediment exposures for these periods were compared.

4.6.1. Total protein

There were no differences between in effects of sediment type or exposure length on total protein in a two-way ANOVA. The greatest range of values found within one group was for null polychaetes (figure 7). *A. marina* in group C during week one had the least variation between individuals.

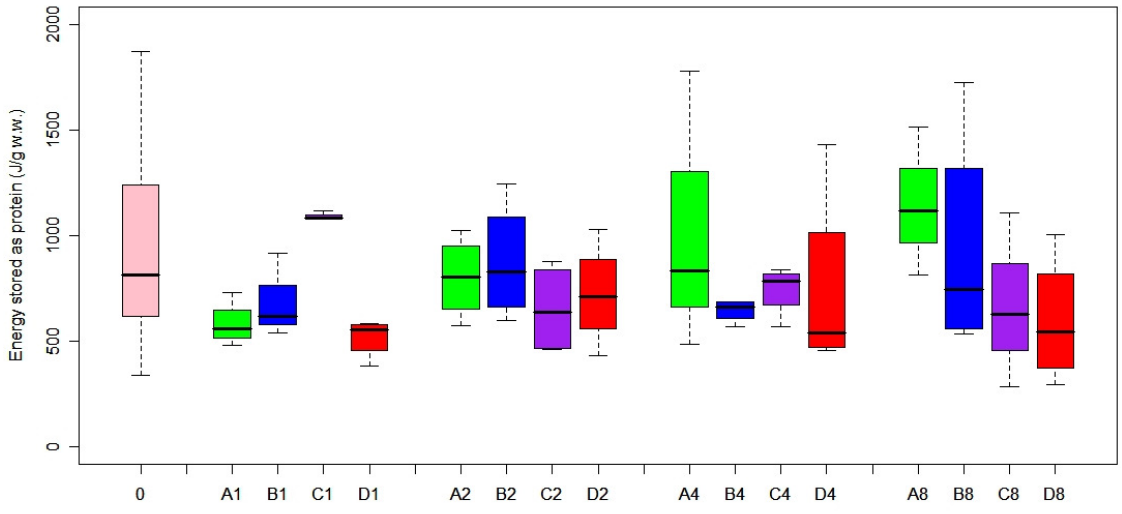

Figure 7. Energy stored as protein in *Arenicola marina* after exposure to test sediments. A, B, C and D indicate exposure sediment. Numbers 0, 1, 2, 4 and 8 indicate the length of exposure in weeks. Median, quartiles, maximum and minimum values are presented.

4.6.2. Differences in total protein during periods

Sediment type was found to have significant effects on the changes in levels of protein within polychaetes in a one-way ANOVA. A Tukey's test found that all exposure groups had protein levels which had affected by different amounts during week 1 (table 9). The greatest median increase was found for group C during this week (figure 8).

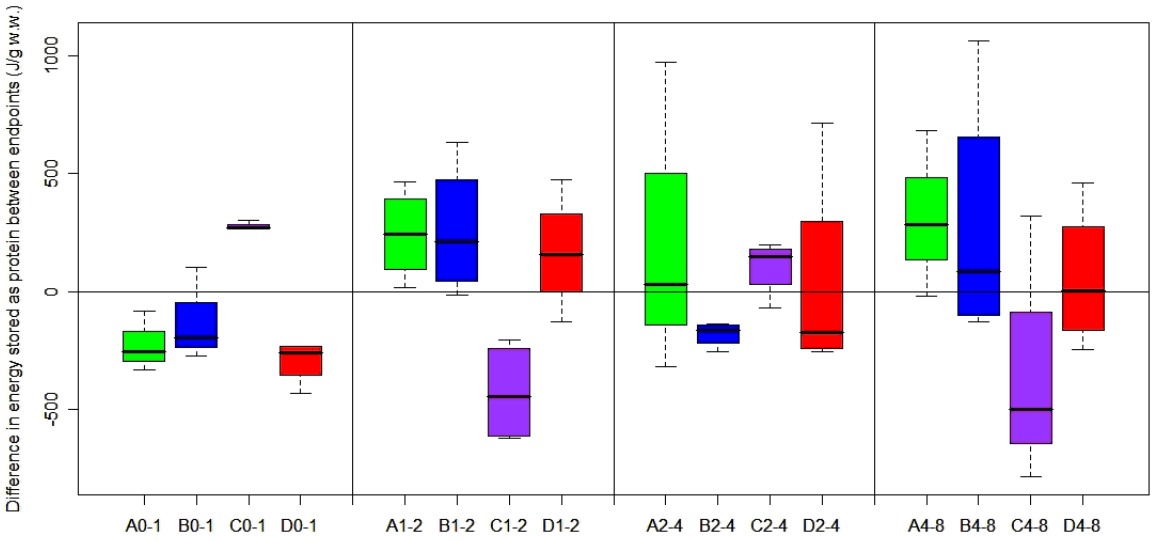

Figure 8. Differences in energy stored as protein in groups (A-D) between endpoints. Calculations were made through subtraction of median for previous endpoint from all values. Median, quartiles, maximum and minimum values are presented.

Table 9. Significant differences between sediments for each week. Results of a Tukey's test following a one-way ANOVA.

Period	Sediments	p
0-1	A and B	0.011
0-1	A and C	0.000
0-1	A and D	0.012
0-1	B and C	0.023
0-1	B and D	0.000
0-1	C and D	0.000

4.6.3. Total carbohydrate

Exposure time and sediment were not found to have any effects on levels of stored carbohydrates in a two-way ANOVA. The greatest variation between individual levels of this energy storage biomolecule for *Arenicola marina* was discovered in the null group (figure 9).

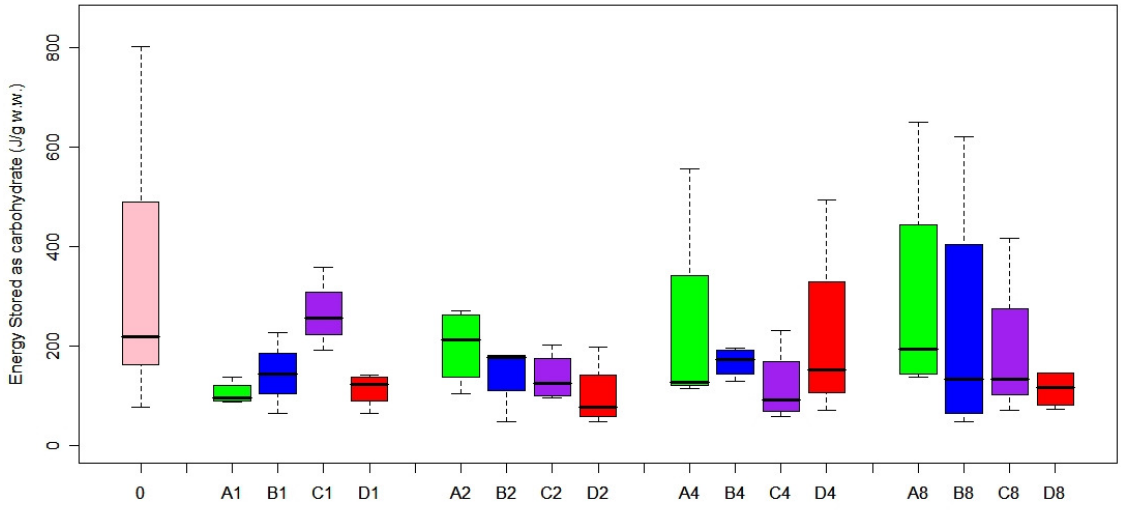

Figure 9. Energy stored as carbohydrates in *Arenicola marina* after exposure to test sediments. A, B, C and D indicate exposure sediment. Numbers 0, 1, 2, 4 and 8 indicate the length of exposure in weeks. Median, quartiles, maximum and minimum values are presented.

4.6.4. Differences in total carbohydrate during periods

Differences between periods for the level of carbohydrates are given in figure 10. Sediment types were seen to have an effect in a one-way ANOVA (table 10). Most significant differences were during the first week, as calculated by a Tukey's test. Sediments A and D both gave different effects than B and C. A difference was detected between control groups A and B during the second week. Sediment A caused a greater decrease in level of carbohydrates than B during the first week but a larger increase during the second week.

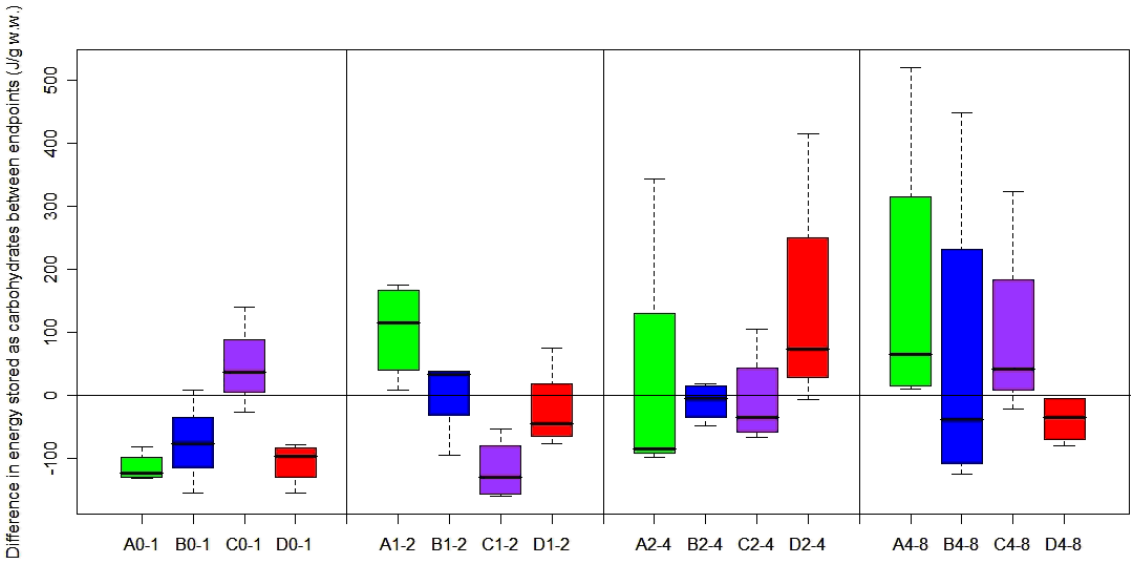

Figure 10. Differences in energy stored as carbohydrates in groups (A-D) between endpoints. Calculations were made through subtraction of median for previous endpoint from all values. Median, quartiles, maximum and minimum values are presented.

Table 10. Significant differences between sediments for each endpoint. The results of a Tukey's test following a one-way ANOVA.

Period	Sediments	p
0-1	A and B	0.017
0-1	A and C	0.001
0-1	B and D	0.006
0-1	C and D	0.001
1-2	A and B	0.041

4.6.5. Total lipid

A significant interaction was found between the sediment and exposure time for the levels of lipids in *A. marina* (p=0.045). During the eight week exposure Sediment A exposed polychaetes lipid concentration is one of the lowest at week. Polychaetes exposed for 8 week have a higher median than all others at weeks 4 and 8 (figure 11). Group C have the highest median level at week one, however, B, C and D are similar from weeks 2 to 8.

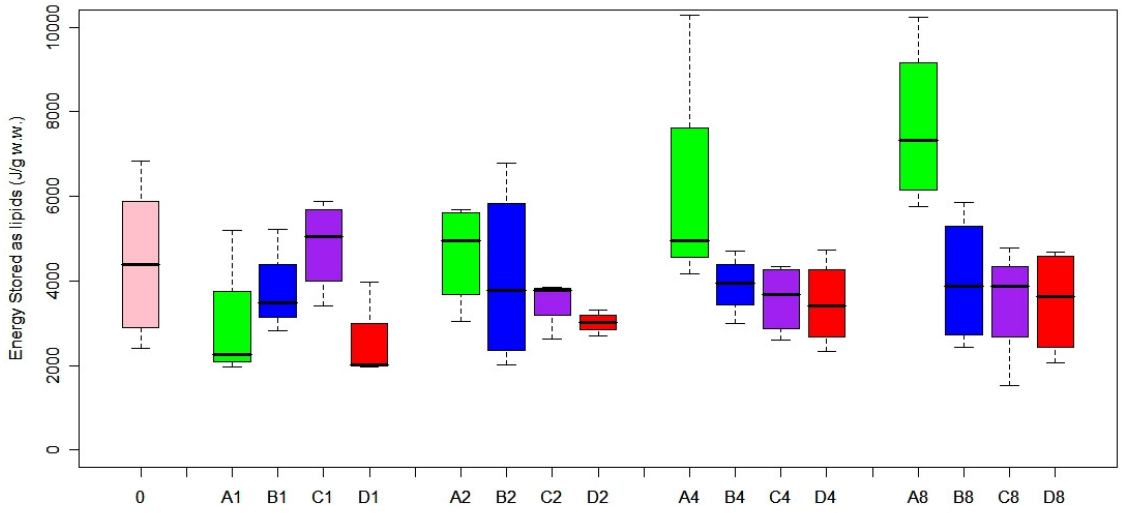

Figure 11. Energy stored as lipids in *Arenicola marina* after exposure to test sediments. A, B, C and D indicate exposure sediment. Numbers 0, 1, 2, 4 and 8 indicate the length of exposure in weeks. Median, quartiles, maximum and minimum values are presented.

4.6.6. Differences in lipid during periods

All significant differences between groups were found during the first and final time periods for changes in energy stored as lipids (table 11). Groups A and D decreased by more than B and C during the first week and C was the only group to have a median gain in lipids (figure 12). During week 8, group A increased in lipid concentration by more than any other group.

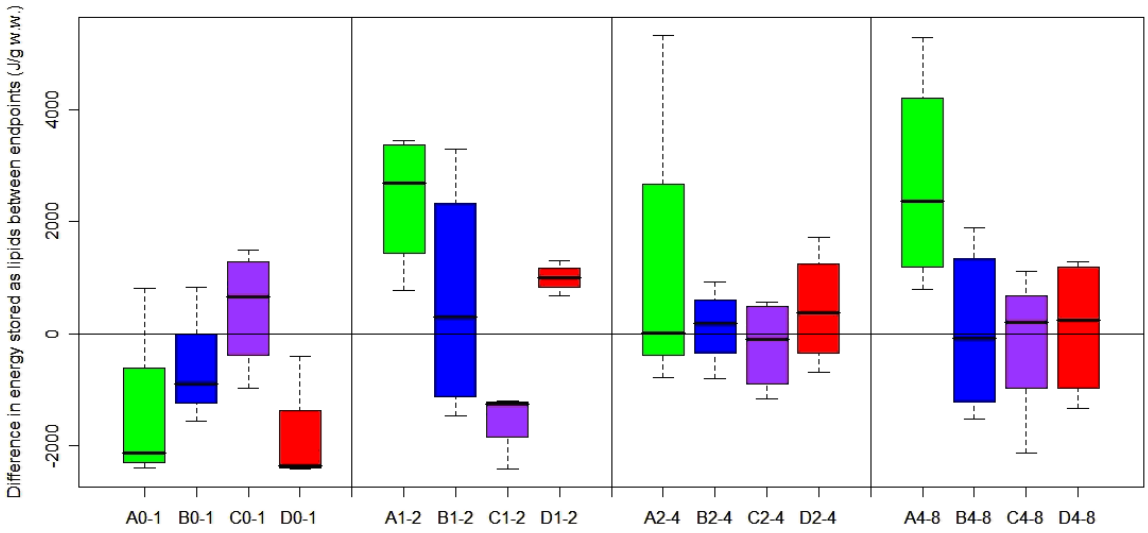

Figure 12. Differences in energy stored as lipids in groups (A-D) between endpoints. Calculations were made through subtraction of median for previous endpoint from all values. Median, quartiles, maximum and minimum values are presented

Table 11. Significant differences between weeks for each sediment and between sediments for each week. The results of a Tukey's test following a one-way ANOVA.

Period	Sediments	p
0-1	A and B	0.018
0-1	A and C	0.025
0-1	B and D	0.002
0-1	C and D	0.003
4-8	A and B	<0.001
4-8	A and C	0.006
4-8	A and D	0.006

4.6.7. Energy available

Trends for available energy are generally the same as those for lipids (figure 13). Sediment type was found to affect the enegy available in a two-way ANOVA (p=0.046). This was revealed to be due to differences between sediments A and D (p = 0.02) by a Tukey's test. Median levels of energy in group A were found at increasingly elevated levels over time relative to sediment D.

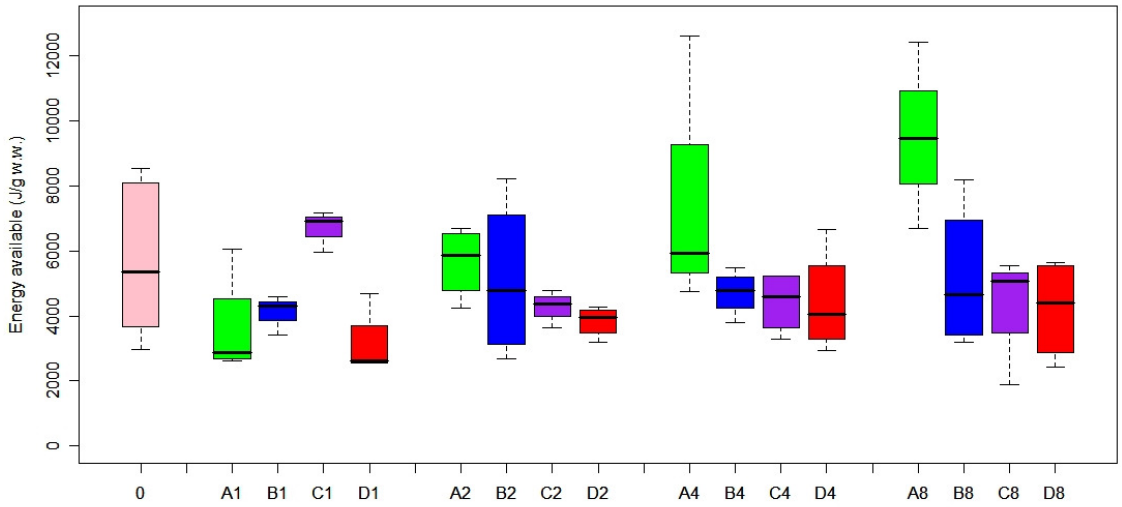

Figure 13. Total available energy in *Arenicola marina* after exposure to test sediments. A, B, C and D indicate exposure sediment. Numbers 0, 1, 2, 4 and 8 indicate the length of exposure in weeks. Median, quartiles, maximum and minimum values are presented.

4.6.8. Differences in available energy (Ea) during periods

Differences in energy levels between sediments were greatest during periods one and four (figure 14). It was determined that Groups A and D lost the largest amounts of energy during the first week at a level lower than both B and C (table 12). Group C had a median increase in energy during this period. During the fourth period, group A increased in levels of energy by more than all other groups.

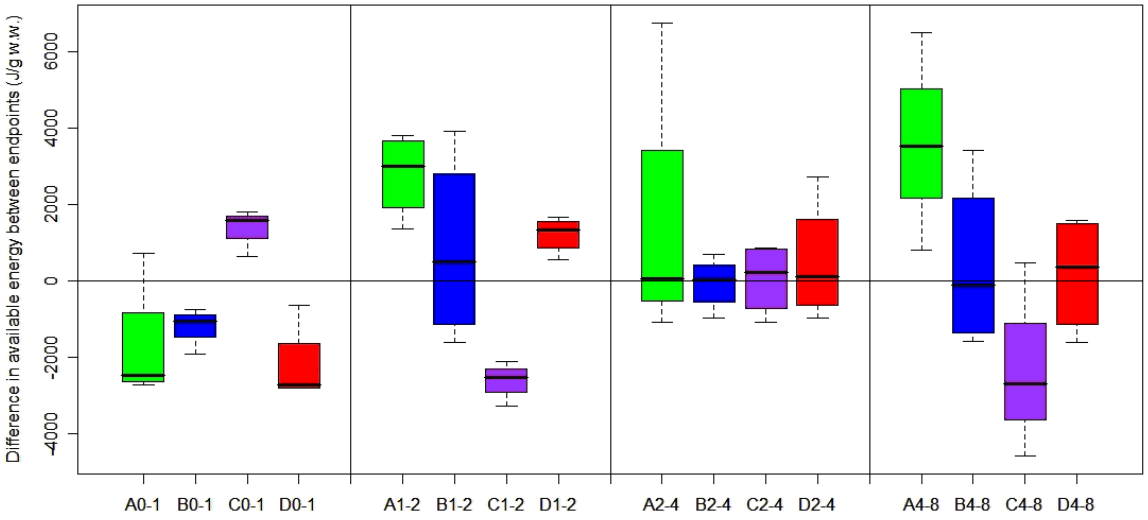

Figure 14. Differences in available energy in groups (A-D) between endpoints. Calculations were made through subtraction of median for previous endpoint from all values. Median, quartiles, maximum and minimum values are presented.

Table 12 Significant differences between sediments for each week. The results of a Tukey's test following a one-way ANOVA.

Period	Sediments	p
0-1	A and B	0.027
0-1	A and C	0.004
0-1	B and D	0.001
0-1	C and D	<0.001
4-8	A and B	0.001
4-8	A and C	0.031
4-8	A and D	0.015

4.6.9. Proportion of the available energy (Ea) made up by each energy source

Proportions of the total stored energy made up by each of the storage biomolecules is given in figure 15. Groups A and D both had initial decreases in the proportion of total energy from lipids from week null to week one to a level lower than B and C. From weeks one to week 8 relative lipid concentrations in each of these groups increased. At week 8 the proportion of available energy comprised of lipids in A and D was greater than in B and C. For each of the treatments the lipids comprised approximately 80% of the stored energy

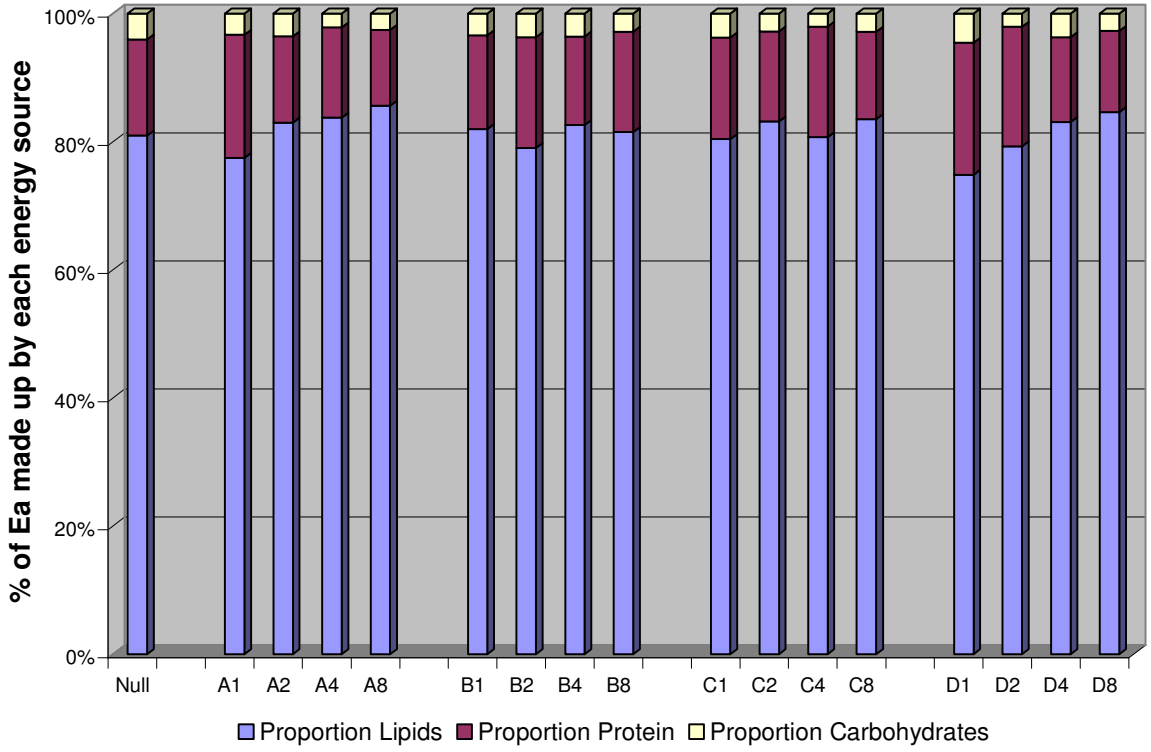

Figure 15. Breakdown of energy sources making up the available energy of each polychaete. Proportions are medians for each treatment. A, B, C and D indicate exposure sediment. Numbers 0, 1, 2, 4 and 8 indicate the length of exposure in weeks.

4.6.10. Energy consumed (ETS)

An interaction factor was found between the effects of sediment type and exposure time for energy consumed in a two-way ANOVA (p=0.06). After one week of exposure the energy consumption per week for sediments B and C were the highest (median values) (figure 16). This trend was further elevated by week 2, but by the final endpoint sediment A had the highest median ETS rate.

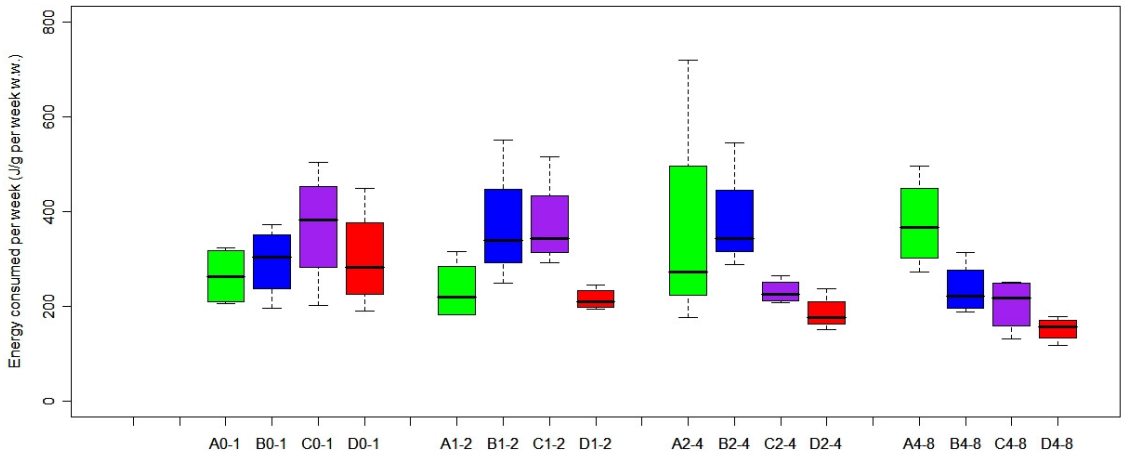

Figure 16. Differences in energy consumed in groups (A-D) between endpoints. Calculations were made through subtraction of median for previous endpoint from all values. Median, quartiles, maximum and minimum values are presented. Note all values are given per week.

4.6.11. Cellular energy allocation

Differences in CEA were found at week one between sediment C and sediments A and D (table 13). Group C was also found to have a higher CEA at week one than all subsequent endpoints. There was a general reduction in the intra-group variation over time (figure 17)

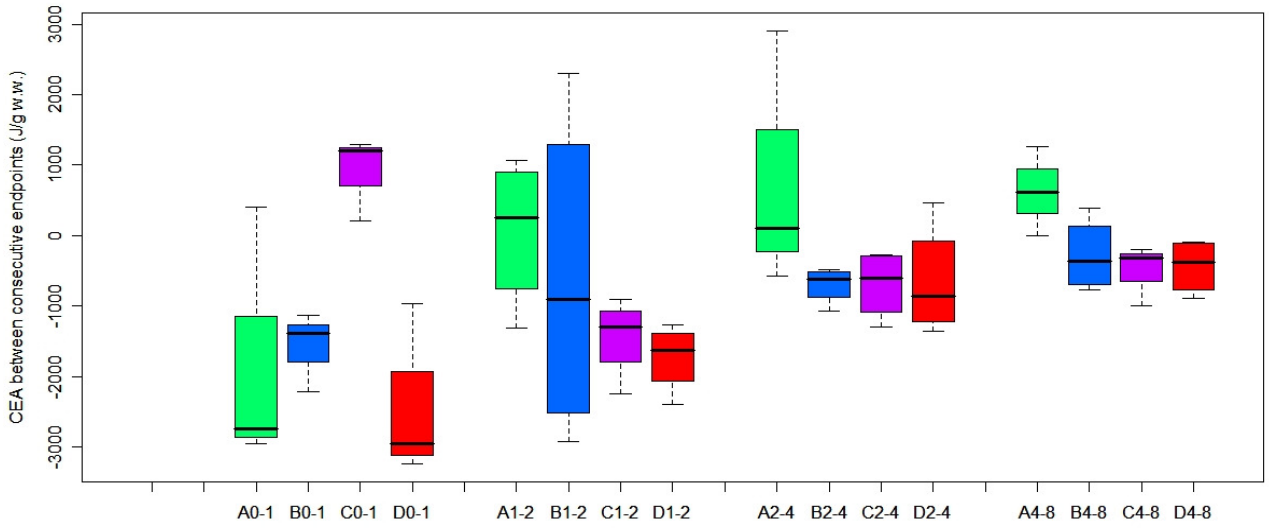

Figure 17. Differences in cellular energy allocation between endpoints. Calculations were made through subtraction of median for previous endpoint from all values. Median, quartiles, maximum and minimum values are presented.

Table 13. Significant differences between sediments for each week (a) and between weeks for each sediment (b). The results of a Tukey's test following a one-way ANOVA.

(a) Week	Sediments	p
1	A and C	0.028
1	C and D	0.011

(b) Sediment	Weeks	p
C	1 and 2	0.001
C	1 and 4	0.012
C	1 and 8	0.036
D	1 and 8	0.018

5. Discussion

5.1. Sediment quality

Differences in suitability were discovered for the different test sediments. Frierfjord and Solbergstand sediments were both mainly comprised of particles less than 63 μm. The majority of particles in Elle sediment were over this size. It is known that the optimum particle diameter for *A. marina* is between 80 μm and 200 μm (Longbottom, 1970). *A. marina* are rarely found in sediments larger or smaller than these sizes. Although the largest particle fraction measured was 'greater than 63 μm' it is known that Elle sediment was closest to the optimum range.

In this study, the Frierfjord sediment had the around twice the organic carbon content of Solbergstrand and more than three times the level at Elle. Longbottom (1970) described a positive relationship between levels of organic carbon and biomass of *A. marina*. The two Frierfjord sediments were thus the most suitable in this aspect of sediment quality and Elle was the least suited.

5.2. Contaminant levels

It was established that levels of estrogenic contamination within the Elle sediment were greater than in other exposure sediments. It has recently been determined that estrogen receptors in polychaetes are activated by similar compounds to those in humans (Keay and Thornton, 2009, Garcia-Alonso et al., 2006). Large differences were also found in the levels of anti-estrogens in Frierfjord sediments. Concentrations in sediments which would affect *A. marina* are not yet known. Further research is needed to determine if the levels of environmental estrogens found in the sediment could have had an effect on the polychaetes.

Sediments C and D were, for each toxicant, found to be more contaminated than the control sediments. For many of the contaminants there was between a two and ten fold higher level in both Frierfjord sediments. For PCB 209 and dioxins however, several hundred times greater concentrations were found at Frierfjord. Nevertheless, results from the bioaccumulation tests indicated lower differences between contaminated and non-

contaminated sediment exposed groups. PCBs were detected only in groups C and D. Differences were found between levels of the metals lead, copper and each of the PAH groups. Frierfjord replicate C exposed *A. marina* had in each case the highest contaminant level. However, Frierfjord D group had similar levels of bioaccumulated metals to control groups (A and B) and lower concentrations of all parameters than group D. It is not possible to explain these results given the data available. One possible reason for these discrepancies can be found in a report of the results of an American monitoring survey (O'Connor et al., 1998). Here it was determined that toxicities of sediments could not be established from chemical analyses alone. A combination of biotic and abiotic factors interact to affect the bioavailabilities of contaminants. These interactions are not yet fully understood.

Interestingly the null sample was found to contain the greatest levels of all of the metal contaminants. Levels for PAH bioaccumulation were also comparable to those of contaminated sediment exposures. Research into the source of the polychaetes revealed that they were bred in heated water which was taken from the vicinity of a coal burning power station. Rain water percolating through a coal storage area, outside the power plant is thought to be the reason for this contamination. Irrespective of this all groups were exposed to the same levels of contamination during breeding. Seabait farmed *Arenicola marina* have also successfully been used in toxicity testing (Hannam et al., 2008). It is therefore expected that differences in subsequent exposure to differing levels of toxic stress would produce relevant results.

5.3. Oxidative stress

The current investigation revealed no differences between any of the sediment exposure groups nor were there any detected between exposure lengths. Significant results have been attained for effects in contaminated aquatic organisms in several studies (Ferrira-Cravo et al., 2007; Regoli, 2000; Regoli et al., 2002; Camus et al., 2002; Benedetti et al., 2009; Winzer et al 2001) whilst other investigations have not found significant effects despite elevated levels of accumulated contaminants (Grung et al., 2009; Camus et al., 2003).

High TCDD levels have been demonstrated to have reductions on TOSC in an investigation on the fish *Trematomus bernacchii* Benedetti et al., (2009). The same investigation however detailed a reduction of these effects in the presence of the heavy metals lead and copper whilst an additive effect was found for combined exposures to TCDD and mercury. Together with high levels of TCDD, heavy metals were detected in each of the exposure groups and exposure sediments in this current study. A lack of response may have been due to interactions between the contaminants within the *A. marina*.

Although it was unknown in the initial stages of this investigation, it has been reported that differences exist in TOSC for different body parts of *A. marina* (Ferreira-Cravo et al., 2007). Relative to the anterior region, the posterior was found to have a greater TOSC for peroxyl and hydroxyl radicals. Nevertheless, the current investigation utilised parts from the posterior and anterior of each specimen. It is expected that this gave a fair representation of the total polychaete TOSC.

Despite no significant results being detected there were slight trends in the data. For all three oxidative stressors the median TOSC values for control sediment B were above the medians of groups C and D in seven out of twelve exposure groups and below C and D only once. This may suggest that an increased sample size could have given more significant results. Polychaetes are however, known to be a pollution tolerant class. They are also the commonest invertebrates found in polluted areas (Eriksen et al., 1988). The lack of any significance during an exposure of this level is an indication that *A. marina* are able to tolerate high levels of oxidants such as those found at in Frierfjord.

5.4. Cellular energy allocation

5.4.1. Weight of *Arenicola marina*

It was determined that weight of A. marina was affected by the sediment type and length of exposure. Elevated weights found in contamination exposed group D by comparison to control group A. This is apparently in contradiction the principle of metabolic costs as proposed by Warren and Davis (1967). Levels of organic carbon were lowest in Elle

sediment and it is possible that this could account for some of the initial differences in biomass.

The effects of the estrogenic contamination could also have caused effects in weight. It is known that female *Hediste diversicolor* can use up to 70% of total somatic energy in the production of gametes (Fidalgo e Costa, 2003). Longitudinal muscles are metabolised to produce oocytes which are stored within the coelomic cavity (Pacey and Bently, 1992; Meijer, 1979). As structural components are removed in this process and replaced by oocytes which displace coelomic fluid, a reduction in weight could be expected. It has also been demonstrated that male polychaetes can synthesize precursors of oocytes proteins in the presence of environmental estrogens (Matozzo et al., 2007). However, at this time it is not known how the concentrations of estrogens within Elle sediment relate to physical changes, if any for *A. marina.*

One further explanation is given by Rasmussen and Andersen (2000). In an investigation of cadmium levels on water balance in *A. marina*, a concentration dependant increase in wet weight was found. It was suggested that cadmium inhibited the production of urine in the polychaete causing swelling. Cadmium levels in sediment D were considerably higher than in A but this relationship is not reflected in bioaccumulated levels, neither are there any apparent effects of cadmium between other groups.

For groups B, C and D weights were found to increase from week one to weeks two and four. Week 8 was not found to be different from week 1. This may be due to initially higher levels of nutrients in the sediment and their subsequent depletion. One interesting result is that group A appear to show the opposite trend at week 4. The drop at an earlier endpoint for Elle sediment exposed polychaetes may be due to lower initial levels of TOC than the other sediments. However, measurements of TOC after the start of the exposure were not made and this hypothesis cannot be proven.

5.4.2. Protein

Whilst there were no detected differences in the absolute levels of protein within *A. marina* for the four exposure groups, it was determined that all treatments during the first period resulted in significant effects. The CEA assay is based on the principle that toxic stress and resultant coping mechanisms have a metabolic cost which leads to changes in the metabolic balance of organisms (De Coen and Jannsen, 1997). It is generally expected that toxic stress will result in reductions of energy containing biomolecules. Nevertheless, there was a greater level of increase of protein in Frierfjord C exposed polychaetes than all other groups during period one. Increases in protein concentration after toxic stress can be explained by an increase in synthesis of detoxification proteins (Devonshire et al., 1998; Smolders et al., 2003). Such synthesis could explain this apparent anomaly.

5.4.3. Carbohydrates

As for protein concentration there were no effects of sediment type on the absolute levels of carbohydrates. However, a comparison of sediments over the first period found that Elle and Frierfjord D sediment exposure had resulted in greater reductions than in groups B and C. It is known that carbohydrates and lipids are the most readily utilised energy sources used by *Daphnia magna* under toxic stress from Zinc (Muyssen, 2002). These biomolecules were therefore assumed to be the most readily available energy sources during toxic stress. While this explains the reduced level of carbohydrates in Frierfjord D polychaetes, the increase in more heavily contaminated Frierfjord C was difficult to interpret. Elle *A. marina* also had a greater drop in carbohydrate level than the Solbergstrand exposed group. During the second period it can be seen that the trend of control sediments is reversed with greater increases of carbohydrates for group A. These fluctuations are an indication that differences at each of these sediments may be related to short term differences rather than long term health effects.

5.4.4. Lipids

The two-way ANOVA for lipids with sediment and exposure time found that there were differences due to sediment type, but also that there were interactions making it difficult

to interpret these results. Interestingly the same trend exists during the first period for lipids as was found for carbohydrates. These results are unexpected for the same reason as those for carbohydrates. In addition it was determined that the lipid concentration was reduced in groups C and D relative to group A polychaetes during the fourth period. Reduced lipid concentration in zebra mussels was also found after exposure to a polluted estuary (Smolders et al., 2004). The metabolic costs of resistance on polychaete lipid levels have been demonstrated by Pook et al. (2009). It was found that *Hediste diversicolor* from a population resistant to heavy metal contamination still had reduced lipid levels after transplantation to a non- contaminated site. Consequently it was deduced that the metabolic cost of an elevated level of detoxification processes produced by these polychaetes caused a reduction in lipids. Differences were also detected between the two control sites for lipids. It is also possible that the estrogenic compounds present in sediment A caused an increase in the production of fat rich vitellins used as energy stores within the oocytes (Garcia-Alonso et al., 2006; Matozzo et al., 2008; Lee et al., 2005). However, as mentioned previously, current knowledge on this aspect of toxicology is not yet at the stage where such a conclusion could be drawn.

Greater increases in lipids relative to Solbergstrand polychaetes is a possible result of the better suited sediment particle size found at Elle. Longbottom (1970) hypothesized that a lack of *A. marina* in finer sediments was due to increased difficulties in maintaining the burrow. While this could explain these findings, *Arenicola marina* are known to tolerate wide range of environmental parameters (Zebe and Schiedek, 1996; Kaag et al., 1998). One of many detected contaminants or an interaction of these could have also been responsible for discrepancies in the weights of the control groups.

5.4.5. Available energy

Approximately 80% of the available energy within *A. marina* was made up by the lipid fraction. As such trends for both parameters are quite similar. One difference was the lack of an interaction between sediment and exposure time for the two-way ANOVA. Sediment type was determined to affect energy levels with Elle sediment exposure causing a greater increase in available energy than the Frierfjord D. Comparisons of

periods revealed exactly the same trend as for lipids. It can therefore be assumed that the balance of lipids is the most important factor for the amount of available energy in polychaetes. In separate studies on *Daphnia magna*, De Coen and Janssen (2003) and Muyssen et al., (2002) both found that lipid budget was the key factor in describing CEA. Both of these studies also found that the energy consumed was a minor factor.

5.4.6. Energy consumed

Energy consumption was affected by sediment although with an interaction factor. An investigation into scope for growth of heavy metal resistant *H. diversicolor* found decreased growth and fecundity and increased respiration compared to non-resistant polychaetes in uncontaminated sediment (Pook et al., 2009). It was thus established that the metabolic costs of toxic stress were met from increases in the demand side of the energy budget. This indicated the energy for detoxification comes from trade offs in aspects related to growth and reproduction and not increases in energy uptake. ETS has been described as an important factor for the determination of CEA in *N. integer* after pesticide exposure, whilst it was found not to influence CEA for *Dreissena polymorpha* after exposure to industrial effluents (Verslycke et al., 2004; Smolders et al., 2004). In general oxygen consumption changes are known to be difficult to interpret due to various biotic and abiotic factors and their interactions (Roast et al. 1999)

In the comparison of changes in available energies and consumed energy, one anomaly was discovered. Median differences for the reductions in available energy during week 1 for sediments A and D and week 2 for sediment C in excess of 2000 J per gram w.w. Respirations measured by ETS never exceed 800 joules per gram per week. Furthermore, ETS is believed to be an overestimation of respiration (Smolders et al., 2004). One possible reason for the lack of significance at this point is a possible time lag between actual changes in the physiological rate of respiration and biochemical levels of ETS, which is based on levels of synthesized enzymes. The length of this lag in *Arenicola marina* is unknown, however, for *Dreissena polymorpha* it was found to be in the range of days to 2 weeks (Smolders et al., 2004). No evidence of delayed increases in

respiration was found during the eight week exposure period. Clearly, not all of the processes of the net energy budget are fully understood.

5.4.7. CEA

For CEA it was determined that group C had elevated levels compared to groups A and D However, high CEA levels for the group C, which bioaccumulated the most contamination, would be in contradiction of the metabolic cost theory. It has been hypothesised that organisms may alter their metabolic strategies in the presence of toxicants in order to achieve maturity more rapidly (Pook et al., 2009; Sibly and Calow, 1989). The manifestation of such a process in *A. marina* has not been discovered but this could explain alterations in the levels of individual energy storage biomolecules. The similar values for group C and other groups in periods 2 to 4 indicated that effects found during the first period may have only been temporary. However, an investigation into the effects of cadmium on *D. magna* it was found that elevated sensitivity on day seven after hatching in comparison with day four (Knowles and McKee, 1987). Reductions in *D. magna* energy reserves were detected but an investigation of the relevance of these findings found that the earlier effects had the greatest significance for the population level. In likeness with these results, the early differences indicated in the CEA of *A. marina* could be of more significance than subsequent results. Further study is required to determine if these effects have any relevance at the population level. Nevertheless the finding of a single period of sensitivity highlights a need for sampling at multiple endpoints in CEA.

For both groups exposed to contaminated sediments, different CEA values were discovered during the final period compared to the first. However, for group C there was a reduction in CEA over time whilst for group D an increase was detected at each subsequent period. Differences in levels of bioaccumulation can explain some differences but it was unexpected that the contaminated groups should show opposite trends.

5.5. Sediments

5.5.1. Replicate contaminated sediments

Frierfjord replicate sediments C and D were reportedly taken from the same area although personal experience of sediment box coring indicates a time of around 15 minutes between box cores. It cannot thus be ruled out that there was some drift in the position of the sampling vessel. Levels of contaminants in sediments C and D were found to be highly similar. Despite this, there were marked discrepancies in concentrations of bioaccumulated metals and PAHs. These differences appear to have caused significant effects at week one for groups C and D. One possible reason for this result is that the slightly different areas within Frierfjord could have large differences in levels of bioavailable contaminants. This difficulty in predicting toxicities from chemical levels is described by Di Toro et al. (1992) and it emphasizes the usefulness of toxicity testing as a tool for environmental monitoring. Variations in replicate samples from a similar location suggest that future exposure sediments should be comprised of multiple box core samples from within a test area.

5.5.2. Control sediments

Differences were detected between the two control sediments as can be expected from two separate sample from different areas. Levels of metals were greater in sediment B but, with the exception of copper, this trend was not repeated in the bioaccumulation results. Despite elevated levels of high molecular weight PAH found in the sediment B, only a small difference was detected in bioaccumulated levels. These differences did appear to have some influence on the effects of available energy although not CEA or TOSC. Nevertheless, these detected variations demonstrate the usefulness of two control samples in place of one. This procedure is suggested for further investigations into the energy budgets of sediment dwelling organisms due to the unpredictability of sediments in toxicity testing.

5.6. Conclusions

Oxidative stress was extensively examined during this investigation with no significant effects found after exposure to high levels of contamination. Interactions between contaminants could be responsible for masking detectable effects. However, the results of this investigation indicate no change in the ability of *A. marina* to scavenge oxyradicals.

During the first week polychaetes with the highest levels of bioaccumulated contaminants stored the most energy. It was unexpected for a contamination exposed group to have a higher CEA than the control groups. Nevertheless, detected alterations in the energy budget of *A. marina* may have implications at the population level.

For both of the replicate Greenland sediments there were differences between calculated CEA values for period 1 and periods 2, 3 and 4. It can thus be concluded that differences exist in the CEA of contamination exposed *A marina* over time. The lack of significant results at during subsequent periods also indicated that week one was the most sensitive period.

A. marina exposed to Frierfjord sediments had lower levels of available energy polychaetes exposed to the control sediment from Elle. This may be caused by the increased metabolic cost of detoxification. The fourth period was the most sensitive with regard to the ability to increases in stored energy

Confounding evidence was found towards the health of Elle exposed polychaetes. Increases in available energy indicate an increased level of health whereas reductions in weight seem to contradict this hypothesis. It is possible that environmental estrogens may be responsible for some of these differences.

Finally, toxic effects detected by the cellular energy assessment methodology were not found in the total oxyradical scavenging capacity for three oxidative sources. It was concluded that effects in the energy budget found in a contaminated sediment sample were not caused by supersession of oxidative stress resistance in *Arenicola marina*.

5.7. Future research needs

During the course of this study, several areas which require further attention emerged. For TOSC and CEA to be applied as useful tools in sediment monitoring investigation into these areas is required.

One interesting result in the current investigation was the increase in CEA of the most contaminated polychaetes. This appears to contradict the concept of the CEA methodology and evidence of an effect was only apparent during one period. Further investigation is required into mechanisms involved for this altered energy balance. In addition the relevance of this effect at population should be determined.

This investigation investigated the effects of contaminated sediments from Frierfjord. It was discovered that two sediments from the same area had different effects on CEA. Furthermore discrepancies in bioaccumulated levels of contaminants from these sediments were detected. Reasons for differences in toxicity of sediments from the same area should be established.

Certain reductions in available energy during the first two weeks exceeded the total respiration by more than a factor of two. It is recommended that investigation be undertaken into whether there is some inaccuracy in the measurement of respiration by ETS in *A. marina*. Energy losses not related to respiration is another possible cause which should be considered.

The effects of environmental estrogens on invertibrates is a new area toxicology. Further research is needed into the effects on *A. marina* of sediment bound estrogenic compounds. If levels of environmental estrogens are found to affect the health of A. marina, an assay for vitellogenin in this species should be developed.

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7. Appendix I: List of reagents and solutions

7.1. Reagents

7.1.1. Reagents for CEA

CEA	Producer	Number
2-(4-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride	Sigma Aldrich	18377
Bovine serum albumin (BSA)	Sigma Aldrich	9048-46-8
Bovine γ globulins	Bio-Rad	500-0005
Chloroform, pro analysi	Sigma Aldrich	132950
DC Protein assay: Reagent A	Bio-Rad	500-0113
DC Protein assay: Reagent B	Bio-Rad	500-0114
Ethylenediaminetetraacetinc acid (EDTA)	Sigma Aldrich	60-00-4
Glycerol, 99%	Sigma Aldrich	56-81-5
Glycogen from bovine liver, type IX	Sigma Aldrich	G0885-1G
Hydrochloric acid, 37%	Sigma Aldrich	320331
Magnesium sulphate heptahydrate	Merk	7487-88-9
Methanol, pro analysi	Sigma Aldrich	322415
Sodium hydroxide	Sigma Aldrich	55881
Phenol, liquefied	Sigma Aldrich	P9346
Polyvinylpyrrolidone	Sigma Aldrich	9003-39-1
Potassium dihydrogen phosphate	Sigma Aldrich	7778-77-0
Potassium hydrogen phosphate	Sigma Aldrich	16788-57-1
Sulphuric acid, 95-97%	Sigma Aldrich	84720
Trichloroacetic acid	Sigma Aldrich	T9159
Tripalmitine	Sigma Aldrich	T5888
Trizma base	Sigma Aldrich	T1503
Trizma hydrogenchloride, reagent grade	Sigma Aldrich	T3253
Triton X-100	Sigma Aldrich	23472-9
β-nicotinamide adenine dinucleotide phosphate reduced form	Sigma Aldrich	N7785-15VL
β- nicotinamide adenine dinucleotide reduced form	Sigma Aldrich	N6785-10VL

7.1.2 Reagents for TOSC

TOSC assay	Producer	Number
α-keto-γmethiolbutyric acid (KMBA)	Sigma Aldrich	K6000
2,2' Azobis (2-methylprpionamideine) dyhydrochloride (ABAP)	Sigma Aldrich	44091-4
L- ascorbic acid (AA)	Sigma Aldrich	A5960
Diethylenetriaminepentaacetic acid free acid (DTPA)	Sigma Aldrich	D6518
Ethylenediaminetetraacetic acid disodium salt (EDTA)	Sigma Aldrich	E1644
Ferric chloride hexahydrate ACS (Fe)	Merck	1039430250
SIN-1 hydrochloride (SIN)	Calbiochem	567028
Glutathione, reduced form, free acid (GSH)	Sigma Aldrich	G4251

7.2 Solutions

7.2.1 Solutions required for TOSC

AA
1.8 mM L- ascorbic acid in 1ml distilled water

ABAP
200 mM 2,2' Azobis (2-methylprpionamideine) dyhydrochloride in distilled water.

DTPA
1 mM Diethylenetriaminepentaacetic acid free acid in distilled water

EDTA
8 mM Ethylenediaminetetraacetic acid disodium salt in phosphate buffer

Fe
4 mM Ferric chloride hexahydrate ACS reagent in distilled water

Fe + EDTA
1. Mix (1:1) of Fe and EDTA
2. dilute (1:111) in distilled water

Homogenisation buffer
100mM KH_2PO_4 buffer pH 7.5, 2.5% NaCl in distilled water

KMBA
2 mM α-keto-γmethiolbutyric acid in distilled water

SIN
800 μM SIN-1 hydrochloride in distilled water

7.2.2 Solutions required for CEA

BSS (buffered substrate solution)
0,1M Trizma HCl/ base buffer pH 7,5, 0.3% Triton X-100

Homogenisation buffer
0.4 M $MgSO_4$, 15% polyvinylpyrrolidone (PVP) and 0.2% (w/v) Triton X-100 in 0.1M Trizma HCl/ base buffer pH 7.5

INT
4mg/ml 2-(4-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride in distilled water

NAD(P)H
1.17 nM NADH, 250µM NADPH in distilled water

15% TCA (trichloroacetic acid in distilled water)

5% TCA (trichloroacetic acid in distilled water)

5% phenol solution in distilled water

7.3. Contents of reaction glasses for TOSC

Volumes of reagents required to test a sample for TOSC against; peroxyl radicals (a), hydroxyl radicals (b) and peroxynitrite anions (c). Volumes are in µl

(a) Peroxyl	-ve Control	+ve Control	Samples
Buffer	800	730	600
Sample	0	0	100
Glutathione	0	70	0
ABAP	100	100	100
KMBA	100	100	100

(b) Hydroxyl	-ve Control	Samples
Buffer	700	600
Sample	0	100
Fe + EDTA	100	100
AA	100	100
KMBA	100	100

(c) Peroxynitrite	-ve Control	Sample
Buffer	700	600
Sample	0	100
DTPA	100	100
SIN	100	100
KMBA	100	100

8. Appendix II: List of samples in each group

	Peroxyl	Hydroxyl	Peroxynitrite	Protein	Carbohydrate	Lipids	ETS
A1	4	4	4	4	4	4	4
A2	4	4	4	4	4	4	4
A4	3	2	3	3	3	3	3
A8	4	4	4	3	4	4	4
B1	2	4	4	3	3	3	3
B2	4	4	4	4	4	4	4
B4	4	4	4	4	4	4	4
B8	4	4	4	4	4	4	4
C1	4	4	4	3	3	3	3
C2	4	4	4	4	4	4	4
C4	4	4	4	4	4	4	4
C8	4	4	4	3	4	4	4
D1	4	4	4	4	4	4	4
D2	2	4	4	4	4	4	4
D4	3	4	4	4	4	4	4
D8	4	4	4	4	4	4	4

Groups missing one sample are coloured yellow, those missing two are coloured red.

9. Appendix III: Temperatures and salinities during the exposure period

Day of exposure	Temperature	Salinity	Day of exposure	Temperature	Salinity
-12	7.5	34.3	23	8.2	34.3
-11	7.5	34.4	24	8.4	34.3
-10	7.5	34.4	25	8.2	34.3
-9	7.5	34.4	26	8.3	34.2
-8	7.5	34.4	27	8.3	34.4
-7	7.6	34.3	28	8.5	34.3
-6	7.6	34.5	29	8.4	34.2
-5	7.7	34.4	30	8.9	34
-4	7.9	34.5	31	8.4	33.8
-3	8	34.5	32	8.1	33.7
-2	8	34.5	33	8.2	33.6
-1	8	34.5	34	8.8	33.5
0	8.1	34.5	35	8.1	33.4
1	8.2	34.5	36	8.1	33.4
2	8.3	34.5	37	8.2	33.2
3	8.1	34.5	38	8.6	32.9
4	8.4	34.5	39	8.5	33.3
5	7.9	34.5	40	8.5	33
6	8.1	34.5	41	8.4	33.4
7	8.6	34.5	42	8.6	33.5
8	8.4	34.5	43	8.7	33.5
9	7.7	34.5	44	8.8	33.3
10	8.4	34.5	45	8.8	33.5
11	8.5	34.5	46	9.2	33.7
12	8	34.5	47	9.1	33.8
13	7.9	34.5	48	9	33.7
14	7.9	34.5	49	9	33.9
15	8.1	34.5	50	8.9	33.6
16	Missing data		51	8.4	33.6
17	Missing data		52	9.1	33.2
18	Missing data		53	9	33.2
19	7.9	34.5	54	8.8	33.5
20	8.1	34.4	55	9	33.4
21	8.2	34.3	56	9.2	33.5
22	8.2	34.3			